# STUDIES ON ZOONOTIC JAPANESE ENCEPHALITIS VIRUS MUAR STRAIN

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# DECLARATION

Except for the assistance outlined in the acknowledgements, I declare that the work described in this thesis is my own work and it was carried out in accordance with the Regulations of the University of Liverpool, United Kingdom. The contents of this thesis have not been submitted for a degree or other qualification at any other university.

# ABSTRACT

Japanese encephalitis virus (JEV) is the most important cause of epidemic encephalitis worldwide but its origin is unknown. Epidemics of encephalitis suggestive of Japanese encephalitis (JE) were described in Japan from the 1870s onwards. Four genotypes of JEV have been characterised and representatives of each genotype have been fully sequenced. Based on limited information, a single isolate from Malaysia, the Muar strain, is thought to represent a putative fifth genotype. I have determined the complete nucleotide and amino acid sequence of the Muar strain and compared it with other fully sequenced JEV genomes. Muar was the least similar, with nucleotide divergence ranging from 20.2 to 21.2% and amino acid divergence ranging from 8.5 to 9.9%. Phylogenetic analysis of the Muar strain revealed that it does represent a distinct fifth genotype of JEV. I elucidated the Muar signature amino acids in the envelope (E) protein, including E327 Glutamine on the exposed lateral surface of the putative receptor binding domain of the E protein, which distinguishes the Muar strain from the other four genotypes. Evolutionary analysis of full-length JEV genomes revealed that the mean (range) evolutionary rate is $4.35 \times 10^{-4}$ ($3.4906 \times 10^{-4}$ to $5.303 \times 10^{-4}$) nucleotides substitutions per site per year and suggests JEV originated from its ancestral virus in the mid 1500s. It is postulated to have originated in the Indonesia-Malaysia region and evolved there into different genotypes, which then spread across Asia. No strong evidence for positive selection was found between JEV strains of the five genotypes and the E gene has generally been subjected to strong purifying selection. The ability of intravenous immunoglobulins (IVIGs) which sometimes are used as supportive treatment for JEV infection to protect against strains of JEV representing the five major genotypes was assessed. Neutralization assays showed IVIGs appear cross-reactive across the five JEV genotypes with effective but lower titers for the Muar strain as well as representatives from genotype IV. Whether there are other strains from genotype V, and what happened to them remains unknown.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| α | Alpha |
| B | Beta |
| γ | Gamma |
| 3D | Three dimension |
| Å | Angstrom |
| A amino acid | Alanine |
| ACIP | Advisory Committee on Immunization Practices |
| *Ae.* | Aedes mosquito |
| AIDS | Acquired immune deficiency syndrome |
| AUAP | Abridged universal amplification primer |
| BBB | Blood brain barrier |
| BEAST | Bayesian evolutionary analysis by sampling trees |
| BHK | Baby hamster kidney |
| BL3 | Biosafety level 3 |
| BP | Base pair |
| BSA | Bovine serum albumin |
| C aminoacid | Cystine |
| C6/36 | Mosquito (*Aedes albopictus*) cell line |
| CL3 | Containment level 3 |
| cDNA | Complementary DNA |
| CDC | Centres for Disease Control and prevention |
| CHIKV | Chikungunya virus |
| CSF | Cerebro spinal fluid |
| CNS | Centeral nervous system |
| CTLs | Cytotoxic T lymphocytes |
| CPE | Cytopathic effect |
| C protein | Capsid protein |
| *Cx.* | Culex mosquito |
| DEN | Dengue |
| DENV | Dengue virus |
| dG | Minimal folding free energy |
| DMEM | Dulbecco's modified eagle's medium |

| | |
|---|---|
| dNTP | Deoxyribonucleotide triphosphate |
| *dN/dS* | Nonsynonymous/synonymous rate ratio |
| D. W. | Distilled water |
| ELISA | Enzyme-linked immunosorbent assay |
| E protein | Envelope protein |
| F81 | Felsenstein 81 |
| FBS | Fetal bovine serum |
| FEL | Fixed effects likelihood |
| FP | Fusion peptide |
| g | Gram |
| G | Genotype |
| GTR | General time reversible |
| H amino acid | Histadine |
| HA | Hemagglutination assay |
| HCV | Hepatitis C virus |
| HI | Hemagglutination inhibition |
| HIV | Human immunodeficiency virus |
| HPD | Highest posterior density |
| i.c. | Intracranial |
| ICTV | International Committee on Taxonomy of Viruses |
| i.d. | Intradermal |
| IFEL | Internal fixed effects likelihood |
| IFN-α | Interferon alpha |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IVIG | Intravenous Immunoglobulin |
| JC | Jukes-Cantor model |
| JE | Japanese encephalitis |
| JEV | Japanese encephalitis virus |
| K2P | Kimura 2-parameter model |
| kb | Kilobase |
| kD | Kilodalton |
| KUNV | Kunjin virus |

| | |
|---|---|
| L | Litre |
| LLC-MK2 | Kidney, Rhesus monkey, Macaca mulatta |
| M | Molar |
| Mabs | Monoclonal antibodies |
| MCC | Maximum clade credibility |
| MCMC | Markov chain Monte Carlo |
| MEGA | Molecular evolutionary genetic analysis |
| MEM | Minimum Essential Medium Eagle, HEPES modification |
| MHC | Major histocompatibility antigen complex |
| µl | Microlitre |
| mm | Millimetre |
| mM | Milimolar |
| mRNA | Messenger RNA |
| MP | Maximum parsimony |
| M protein | Membrane protein |
| MRI | Magnetic resonance imaging |
| MVE | Murray Valley encephalitis |
| MVEV | Murray Valley encephalitis virus |
| N amino acid | Asparagine |
| Na Cl | Sodium chloride |
| Na HCO3 | Sodium bicarbonate solution 7.5% |
| NCR | Non coding region |
| NJ | Neighbor joining |
| nm | Nanometre |
| NS | Non-structural |
| NSAID | Non steroidal anti-inflammatory drug |
| NT | Neutralization |
| NTPase | Nucleotide 5`-triphosphatase |
| NVSI | National Vaccine and Serum Institute |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PFU | Plaque forming unit |
| prM protein | premembrane protein |
| $PRNT_{50}$ | 50% plaque reduction neutralization test |

| | |
|---|---|
| Q amino acid | Glutamine |
| RACE | Rapid amplification of cDNA ends |
| RdRp | RNA-dependant RNA polymerase |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| rpm | Revolutions per minute |
| RT | Reverse transcription |
| RTPase | 5`-terminal RNA triphosphatase |
| S amino acid | Serine |
| s.c. | Subcutaneous |
| SLAC | Single likelihood ancestor counting |
| SLE | St. Louis encephalitis |
| SLEV | St. Louis encephalitis virus |
| spp. | Species |
| $T_a$ | Annealing temperature |
| TAE | Tris-Acetate-EDTA |
| TBE | Tick borne encephalitis |
| TBEV | Tick borne encephalitis virus |
| TNF-$\alpha$ | Tumour necrosis factor alpha |
| $T_m$ | Melting temperature |
| ORF | Open reading frame |
| US | United States |
| USU | Usutu virus |
| PAUP | Phylogenetic analysis using parsimony |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase chain reaction |
| PHK | Primary hamster kidney |
| PHYLIP | Phylogenetic inference package |
| P/S | Penicillin/Streptomycin solution stabilised |
| PP | Pooled plasma |
| Vero | Kidney epithelial cells from an African green monkey |
| WBC | White blood count |
| WHO | World Health Organization |
| WN | West Nile |

| | |
|---|---|
| WNV | West Nile virus |
| WRAIR | Walter Reed Army Institute of Research |
| YF | Yellow fever |
| YFV | Yellow fever virus |

# CHAPTER 1: INTRODUCTION

## 1.1 General Introduction to Japanese Encephalitis Virus (JEV)

Flaviviruses are important human pathogens; they include a number of emerging and re-emerging pathogens such as Japanese encephalitis virus (JEV), West Nile virus (WNV), St. Louis encephalitis virus (SLEV), Kunjin virus (KUNV), Murray Valley encephalitis virus (MVEV), yellow fever virus (YFV) and dengue viruses (DENV) causing a variety of diseases ranging from mild febrile illness to severe encephalitis and hemorrhagic fever (Mishra and Basu, 2008).

The most important cause of epidemic encephalitis worldwide is JEV, with an estimated 35,000 to 50,000 cases and 10,000 deaths annually (Tsai, 2000). The magnitude of the problem is even more alarming since a high proportion of survivors have serious and permanent neuropsychiatric sequelae, especially in children (Kaur and Vrati, 2003).

JEV belongs to the Japanese encephalitis (JE) serogroup of the genus *Flavivirus*, family *Flaviviridae* and is transmitted in nature by mosquitoes between vertebrate hosts including birds and pigs (Yun et al., 2003). The risk of JE increases as the number of vector mosquitoes increases. Because the rice paddy-breeding *Culex* mosquitoes are unavoidable, the majority of the population in rural Asia has been infected with the virus by early adulthood. Humans become infected during the bite of an infected mosquito. The expansion of the JE-endemic area depends on increasing irrigated rice field and pig farming. These environments that are suitable for the infectious cycle of JEV exist widely in Asia (Dutta et al., 2011; Oya and Kurane, 2007).

Following infection with JEV, the ratio of symptomatic to asymptomatic disease is estimated to be 1:50-1:300 (Libraty et al., 2002a). JEV targets the CNS causing encephalitis as well as meningitis (Hosokawa et al., 2007) clinically manifesting with fever, headache, vomiting, signs of meningeal irritation and altered consciousness (Kumar et al., 1991). Clinical variables reported to be associated with a poor outcome include depth of coma, extremes of age, hyporeflexia, decerebrate posturing and apnoea attacks, increased febrile response, and elevated cerebrospinal fluid (CSF) pleocytosis, seizures or a polio-like flaccid paralysis (Dapeng, 1995; Hoke et al., 1992; Huy et al., 1994; Kumar et al., 1990; Misra et al., 1998; Schneider et al., 1974; Solomon et al., 2002; Solomon et al., 1998).

JEV was first isolated in Japan in 1935 (Lewis et al., 1947). Subsequently, JEV has been found throughout most of Asia and the virus was most recently isolated in Papua New Guinea and the Torres Straits of Australia, indicating that this virus could become a worldwide public health threat (Hanna et al., 1999; Hanna et al., 1996; Mackenzie et al., 1994).

the origins of the virus are uncertain, but phylogenetic comparisons with other flaviviruses suggest it evolved from an African ancestral virus, perhaps as recently as a few centuries ago (Gould, 2002).

Two distinct epidemiological patterns of JEV transmission have been observed. In northern temperate regions of Asia JE occurs in summer epidemics, whereas in southern tropical areas the disease is endemic and occurs year-round (Vaughn and Hoke, 1992).

## ***Genetic Studies Reveal Different JEV Genotypes***

Based on genetic studies, JEV consists of four genotypes (GI, II, III and IV) (Chen et al., 1992; Chen et al., 1990). Genotype I includes isolates from Northern Thailand, Cambodia, Korea, China, Japan, Vietnam, Taiwan, and Australia from 1967 and the present, Genotype II includes isolates from southern Thailand, Malaysia, Indonesia, Papua New Guinea and Northern Australia before 1951 to 1999, Genotype III includes isolates from mostly temperate regions of Asia, including Japan, China, Taiwan, the Philippines, and the Asian subcontinent from 1935 to the present, and Genotype IV includes isolates from Indonesia between 1980 and 1981 only (Schuh et al., 2010). A representative of each of these four genotypes has been fully sequenced (Solomon *et al.*, 2003b). In addition, a strain of JEV, Muar strain, isolated in Singapore in 1952 from a brain of a patient who originated in Muar, Malaysia (Hale et al., 1952) may represent a fifth genotype as determined by monoclonal antibodies (MAbs) reactivities (Hasegawa et al., 1995; Kobayashi et al., 1984) and limited phylogenetic analysis depending on E gene sequence (Hasegawa et al., 1994; Solomon et al., 2003b; Uchil and Satchidanandam, 2001).

Therapy for JE is supportive and no clearly effective specific antiviral agents exist (Mishra and Basu, 2008). There have been only two placebo-controlled trials in JE which showed no benefit associated with the administration of corticosteroids (dexamethasone) or

interferon alpha (IFN-α) (Hoke et al., 1992; Solomon et al., 2003a). However, there have been case reports of intravenous immune globulin (IVIG), which has been shown to be of benefit when administered with symptomatic treatment for this disease. In the case report, clinical improvement was rapid and the patient made an almost complete recovery (Caramello et al., 2006). Vaccination remains the most promising tool to reduce this deadly infection (Tsai, 2000).

## 1.2 History of JE

Encephalitis due to JEV was described as early as 1870 in Japan but the first described large epidemic occurred in 1924 (Pond and Smadel, 1954). JEV was first isolated in 1934 from the brain of a fatal case of encephalitis and was characterized as the prototype (Nakayama) strain of JEV (Mitamura et al., 1936; Monath, 1988).

Observations of JE epidemics suggested that it was spread by a mosquito vector and had a seasonal disease occurrence. JEV was first isolated from *Culex tritaeniorhynchus* mosquitoes in 1938 (Mitamura et al., 1938). It was over 20 years later that the transmission cycle of JEV between viremic swine and birds to man, an incidental dead-end host, by the mosquito vector *Culex tritaeniorhynchus* was described (Buescher et al., 1959; Scherer, 1959).

## 1.3 Epidemiology of JEV

### 1.3.1 Geographic Distribution and Seasonal Occurrence

JEV has been found throughout most of Asia with reported cases of JE in Thailand, India, Nepal, Bangladesh, Sir Lanka, Vietnam, Burma, Laos, Cambodia, Malaysia, Singapore, Taiwan, Philippines, Indonesia, China, Siberia, Korea, and Japan (Tsai and Yu, 2000).

JEV could become a worldwide public health threat as it was most recently isolated in Papua New Guinea and the Torres Straits of Australia (Hanna et al., 1999; Hanna et al., 1996; Mackenzie et al., 1994). Figure 1-1 shows the current geographical distribution of JEV.

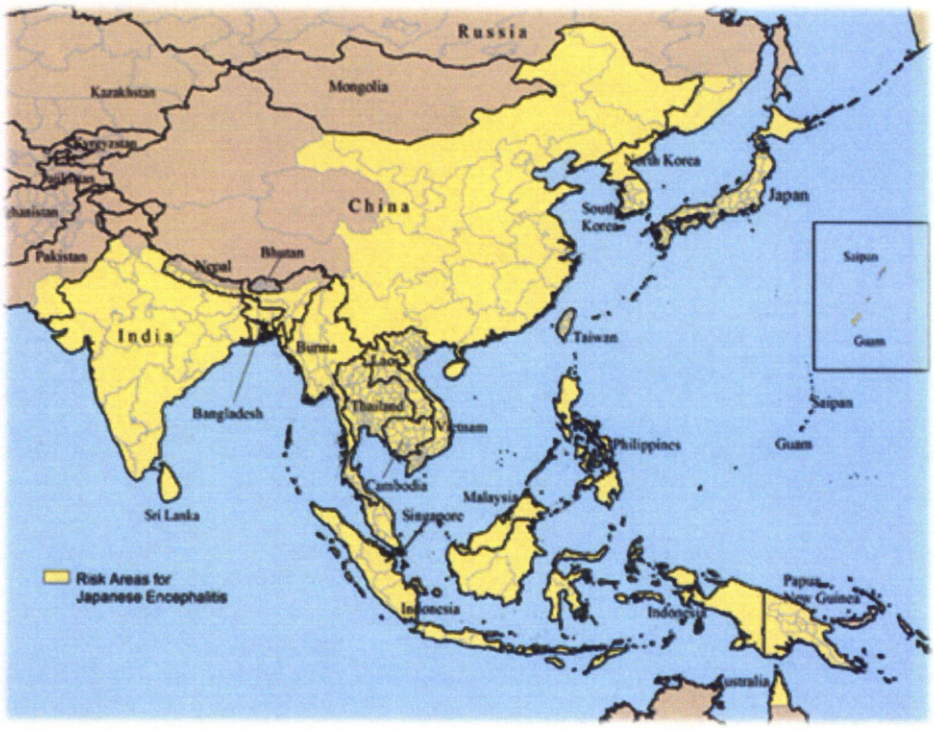


Figure 1-1. Approximate geographic distribution of Japanese encephalitis virus. Yellow indicates areas current or previous epidemics. Taken from Centers for Disease Control and Prevention (CDC). http://www.cdc.gov/ncidod/dvbid/jencephalitis/map.htm

JE incidence in humans is seasonal and varies by country. This is probably due to irrigation practices, ambient temperature and the migration pattern of susceptible birds. For example, in the temperate climates of Korea and Japan, JE occurs from May to September while it occurs from April to October in the tropical countries of Southeast Asia such as Thailand, Cambodia and Vietnam, and from September to December in Nepal and Northern India (Endy and Nisalak, 2002).

### 1.3.2 Transmission Cycle

JEV is a mosquito-borne flavivirus. It is transmitted between vertebrate hosts by mosquitoes, principally by *Culex species* and in particular *Culex tritaeniorhynchus* (Yun et al., 2003). JEV is maintained in nature mainly between vector mosquitoes and animal vertebrate hosts such as pigs and water birds such as egrets and herons.

The primary cycle of transmission and environmental maintenance of JEV depends on a cycle of mosquito infection and vertical transmission to its offspring, infection in water birds and pigs, and further infection in uninfected mosquitoes (van den Hurk et al., 2009).

Swine and water birds develop high levels of viremia upon JE infection, which serves as a viral amplification stage and enhances transmission to mosquitoes that results in further perpetuation of this enzootic cycle. Humans and horses may develop asymptomatic infection or severe encephalitis when infected by JEV following mosquito bite. Humans and horses are considered dead-end hosts as the level of viremia is insufficient for further infection of the mosquito vector and transmission to other susceptible hosts (Endy and Nisalak, 2002). The transmission cycle of JEV is illustrated in Figure 1-2.

Other vertebrate hosts can be naturally infected with JE and include the domestic vertebrates donkeys, chickens, ducks, water buffalos, cattle, and sheep as well as vertebrates in nature to include mice, bats, snakes and frogs (Shortridge et al., 1977). It is not known what role these hosts play in maintaining JEV in nature.

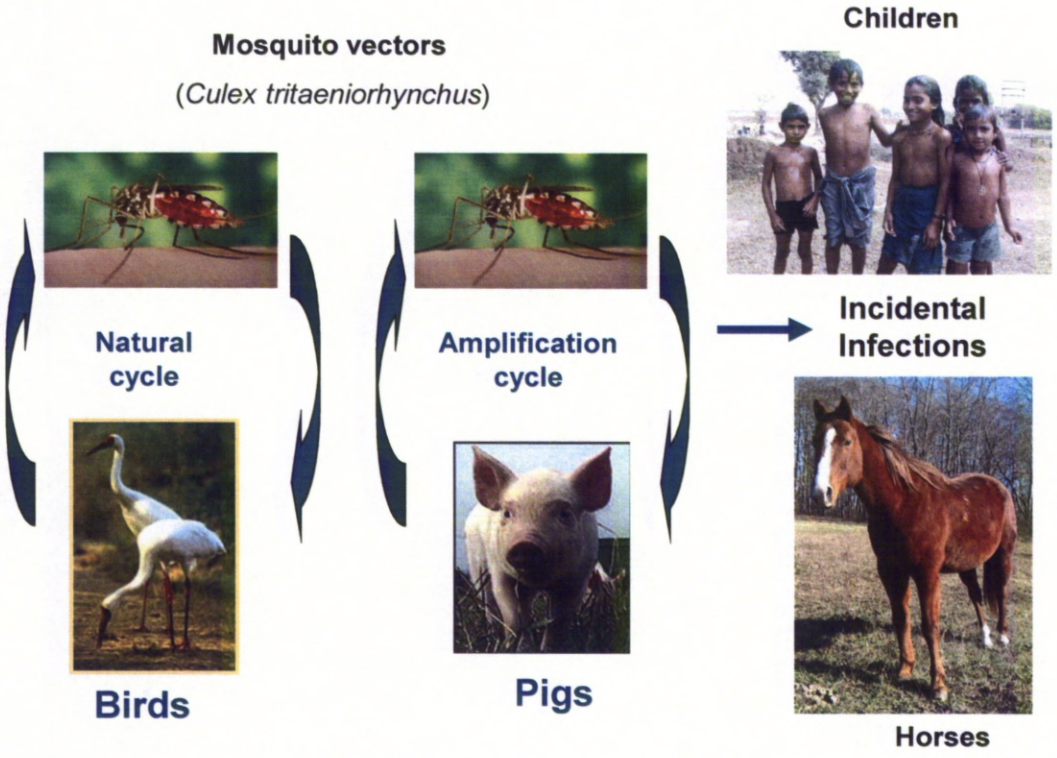


Figure 1-2. Transmission cycle of Japanese encephalitis virus. Adapted from (Misra and Kalita, 2010).

### 1.3.3 Vectors

In field studies, it has been demonstrated that JEV can infect and be transmitted by wide range of mosquito species including *Cx. tritaeniorhynchus*, *Cx. gelidus*, *Mansonia* spp., *Aedes curtipes*, and mixed *Anopheles* spp. (Simpson et al., 1970a; Simpson et al., 1974; Simpson et al., 1970b; Simpson et al., 1976).

In addition to *Cx. tritaeniorhynchus*, JEV has been isolated from other mosquito species such as *Ae. subpictus*, and *Cx. whitmorei* in southern India; *Anopheles hyrcanus* group and *An. barbirostris* in Pakistan; *Ae. albopictus*, and *Armigeres obturans* in China; *Cx. fuscocephala*, and *Mansonia uniformis* in Sri Lanka (Gajanana et al., 1997; George et al., 1987a; Huang, 1982; Okuno et al., 1973; Peiris et al., 1992).

In laboratory studies, it has been demonstrated that JEV can infect and be transmitted by a wide range of mosquito species which are considered to be the most competent vectors in transmitting JEV (Jeong et al., 2011). Certain mosquito species are competent in JE transmission but with lower efficiency including *Cx. pipiens pallens, Cx. p. quinquefasciatus, Cx. pseudovishnui,* and *Anopheles tessalatus* (Burke and Leake, 1988; Gresser et al., 1958; Mourya et al., 1991). Under laboratory condition, *Aedes* spp. are considered to be the least efficient in JEV transmission (Endy and Nisalak, 2002).

Vector competence studies showed the importance of *Cx. tritaeniorhynchus* in JEV transmission. One hundred percent infection rates of laboratory-raised *Cx. tritaeniorhynchus* are observed (Hill, 1970; Soman et al., 1977; Takahashi, 1976).

JEV replicates rapidly in *Cx. tritaeniorhynchus* mosquitoes and viral kinetics, viral load, and transmission are dependent on the ambient temperature (Weng et al., 2005). For example, Maximal titers of virus are reached within 5 days in mosquitoes kept at 28 °C (Takahashi, 1976).

JEV is maintained in mosquitoes through four important mechanisms: overwintering, transovarial infection, infection at oviposition, and sexual transmission (Makiya et al., 1985). Overwintering of JEV has been demonstrated in *Cx. tritaeniorhynchus* (Hayashi et al., 1975; Lee, 1971; Ura, 1976). Transovarial transmission of JEV can occur in *Cx. tritaeniorhynchus,*

(Rosen et al., 1989; Rosen et al., 1978; Soman and Mourya, 1985). Infection at oviposition has been demonstrated to occur in *Cx. pipiens*, *Ae. alcasidi*, and *Ae. flavus* (Rosen et al., 1989). Furthermore, JEV was sexually transmitted from male to female *Cx. tritaeniorhynchus* (Mourya and Soman, 1999; Rosen et al., 1989).

Determinants in JEV epidemiology of human infection include the behavioural characteristics of the primary mosquito vector such as host preference, time of day of feeding, feeding behaviour, and ideal environmental conditions for breeding (Gould et al., 1974).

### 1.3.4 Vertebrate Hosts

#### *Domestic Pigs*

Pigs play an important role in the JEV transmission cycle. Most domestic pigs can develop viremia capable of infecting mosquitoes (Chen and Beaty, 1982). Field studies showed that when JE naive swine are placed into a JE endemic area, they will develop infection within one week (Unni et al., 2011). Swine develop a viremia lasting for four days (Komada et al., 1968; Maeda et al., 1978; Scherer, 1959).

Domestic pigs have been used as sentinel population to monitor and estimate the environmental burden of JE due to the susceptibility of pigs for JEV infection and its preference as a blood meal for the mosquito vector. For example, In India, 18.5% of sentinel pigs the Kolar district, Karnataka, demonstrated seroconversion to JE with isolation of JEV from several sentinel pigs and hemagglutination inhibition (HI) antibodies persisted for 3 years in these pigs (Geevarghese et al., 1987; Geevarghese et al., 1991).

In JE endemic areas free of human epidemic JE, pigs have been used to measure the intensity of environmental transmission of JE (Burke et al., 1985d). In a 5-year survey of JE antibody in swine located in the Natori District of Miyagi Prefecture, Japan, a 100% infection rate of swine was observed even in the absence of human cases (Yamamoto et al., 1970). The appearance of rising antibody and the duration of high-titered antibody were associated with the intensity of JE transmission from mosquitoes and correlated with a higher incidence of human disease (Endy and Nisalak, 2002).

It is not known, how JEV changes at a molecular level during swine infection and within the enzootic cycle of JEV between birds and pigs and its transmission to humans. Also, the effects of interspecies transmission of JE from an avian host to swine and back again on mutation rates and recombination events are not known. However, it is known for other viruses that have an avian-swine transmission, such as the influenza virus, that swine play an important role in the recombination events of this virus and its ability to infect humans (Stech et al., 1999). Transmission from avian to swine hosts may induce an increase in mutation rates (Stech et al., 1999). Pigs are susceptible to infection with avian influenza due to a receptor similar to that found in birds. Following infection, swine act as a mixing vessel that allows recombination events to occur. Swine influenza viral replication then allows reassortment and adaptation of the influenza virus and emergence of a dominant strain that is able to infect humans (Webby et al., 2000). There is no doubt that for JEV reassortment is not possible, because flaviviruses have just a single piece of nucleic acid.

Swine are a major source of food and income in most of Asia (http://www.pigprogress.net). Swine production and the production of newborn and susceptible litters produce a steady supply of susceptible hosts for JEV infection. JE will remain a part of the environment in these countries and as pig production increases, the environmental burden of JE will increase as well as its potential to infect humans (Endy and Nisalak, 2002).

Swine are the preferred hosts for *Cx. tritaeniorhynchus*, followed by birds and then humans (Scherer, 1959). Pigs play an important role in the transmission and maintenance of JEV in the environment as well as for human infection.

### *Avian Vertebrates*

The role birds play in JEV transmission has been demonstrated. High levels of viremia can be produced in large water birds such as the Black-crowned night heron (*Nycticorax nycticorax*), egrets (*Ardea spp.)* as well as Pond herons (*Ardeola grayii*) (Scherer, 1959).

Night herons, Plumed egret and domestic chickens have a high seroprevalance for JE antibody and they are considered sources of JE transmission (Ogata et al., 1970).

### *Horses*

Horses can be infected with JEV and develop CNS infections, and at times precede human clinical cases (Paterson et al., 1952). The clinical signs of JE in horses are similar to human disease (Paterson et al., 1952). Viremia in horses develops from 1 to 4 days after infection, lasting 2-6 days. However, horses, like humans, represent a dead-end host for JE transmission (Gould et al., 1964).

In Japan, JE incidence declined in horses from the initial 0.3 per 100,000 in 1960 to 0.03 per 100,000 in 1967 (Nakamura, 1972). This decline was attributed to the increase in numbers of vaccinated horses with an inactivated JE vaccine (Endy and Nisalak, 2002).

### *Other Vertebrates*

Other vertebrate-hosts could play a role in the JEV transmission cycle. The highest antibody seroprevalence observed was in swine (88.1%), followed by buffalo (45%), cattle (42%), sheep (17.9%), and goats (13.8%) (Oda et al., 1996).

Domestic livestock other than swine appear to have the ability to be infected by JE with low levels of viremia and do not contribute to JE transmission cycle in nature and are considered to represent dead-end hosts (Gibbs, 1976).

Significant antibody titres to JEV have been found in cattle and goats and have been correlated to the occurrence of human diseases (Peiris et al., 1993). Cattle can be infected by JEV with the development of JE antibody, but demonstrate no evidence of viremia (Ilkal et al., 1988). In Japan, 63.6% of new-born calves had maternally derived JE antibody (Horimoto and Sakai, 1990; Horimoto et al., 1987).

Overwintering of JEV has been reported to occur in snakes, frogs, and bats (Oh et al., 1974). For example, insectivorous bats, microchiroptera, can be infected under laboratory conditions with JEV and have demonstrated persistence of viral infection under low environmental temperatures (Sulkin et al., 1970; Sulkin et al., 1966a; Sulkin et al., 1966b). Sera from 626 bats of seven different species in Karnataka State, India were tested for JE antibody (Banerjee et al., 1988). Five species of bats tested positive for JE antibody (7.3%

seroprevalence). This was similar to rates observed in Japan (Miura et al., 1970). However, the role of reptiles, amphibians, and bats in the overwintering of JEV and their contribution to its environmental maintenance is not known.

Rodents are tolerant to JEV infection and in ecologic studies have had no antibodies against JE (Scherer, 1959; Williams and Imlarp, 1972).

### 1.3.5 Environmental Factors

#### Climate and Weather

Climate and weather are important determinants in the environmental maintenance and transmission of JEV to humans. Ambient temperature is the important factor in mosquito vector infectivity and maintenance (Mogi, 1983).

It has been observed that large epidemics occurred during unseasonably dry, hot summers while, wet, cool summers were associated with little human infection (Endy and Nisalak, 2002).

Mosquitoes are extremely sensitive to the climate. For example, meteorological conditions have effect on vector reproduction, mortality rates, blood feeding frequency of the vector and extrinsic incubation period of the viral pathogen (Hay et al., 2000; Kuno, 1997; Lindsay and Birley, 1996).

To demonstrate the climate effect on one mosquito species, a survey of *Cx. tritaeniorhynchus* population from 38 geographical sites throughout Asia was conducted (Reisen et al., 1976). *Cx. tritaeniorhynchus* peaked from June to September in Pakistan following the annual planting of rice fields. In India, *Cx. tritaeniorhynchus* breeding continued throughout the year and peaked during September to December. In Thailand, population trends paralleled the seasonal rainfall pattern with peaks occurring during breeding throughout the year with a peak during October and November. Okinawa, Japan, and Korea all had similar population dynamics with peaks occurring during June to August (Endy and Nisalak, 2002).

## 1.4 Molecular Epidemiology of JEV

### 1.4.1 The JE Serocomplex

Depending on cross-neutralization tests, the *Flavivirus* genus of the family *Flaviviridae* has been divided into eight serocomplexes, including TBE serocomplex, the DEN serocomplex, and the JE serocomplex, with some viruses, including the prototype virus, YFV, not falling into any of these serocomplexes (Calisher et al., 1989; De Madrid and Porterfield, 1974).

The JE serocomplex includes members from across the world, such as MVEV, WNV, KUNV and SLEV (Chamberlain, 1980; Kimura-Kuroda and Yasui, 1986; Porterfield, 1980). JEV is found primarily in Asia whereas WNV is found in Africa and north into Europe, and more recently in North America. SLEV is endemic to North America whereas MVEV and KUNV are found in Australia.

JE serocomplex viruses were classified based on examination of the serological differences among viruses isolated from various regions throughout Asia (Gould, 2002). Strain variation among JEV isolates was first reported by Hale and Lee in 1954 who compared six Malaysian isolates of JEV 'types' (Hale and Lee, 1954). Antigenic variation among JEV isolates based on biological tests using polyclonal antisera, such as HI and neutralization (NT), have been described by others (Banerjee, 1986; Huang, 1982).

Two major immunotypes have been differentiated (Okuno et al., 1968). Using polyclonal antiserum, four 'subtypes' of JEV from northern Thailand were identified on the basis of HI and NT tests (Ali and Igarashi, 1997; Ali et al., 1995).

Polyclonal antisera are clearly able to distinguish separate JEV immunotypes. However, using MAbs allowed a more precise delineation of JEV strains, and several studies have used MAbs generated against one strain of JEV to examine cross-reactivity with other strains.

It has been demonstrated by two laboratories that at least five antigenic subgroups of JEV have circulated in Asia since isolation of the original Nakayama virus in 1935 (Hasegawa et al., 1994; Kedarnath et al., 1986; Kobayashi et al., 1984; Kobayashi et al., 1985). One of these subgroups includes only the Nakayama-derived strains, whereas the majority of the

more recent isolates are within a subgroup containing the Kamiyama strain isolated in 1966 (Kobayashi et al., 1985). In addition, two serological subgroups are quite distinct, the 691004 (isolated in Sri Lanka in 1969) and Muar (isolated in Singapore in 1952) subgroups, each containing only the prototype virus for the respective subgroup (Kobayashi et al., 1985).

Examination of 11 geographically diverse JEV strains suggests at least five epitopes associated with HI activity (Kobayashi et al., 1985) whereas examination of a combination of HI, NT, and enzyme-linked immunosorbent assay (ELISA) data suggests up to eight distinct epitopes (Ghosh et al., 1989; Kimura-Kuroda and Yasui, 1983, 1986). JE-specific HI and NT epitopes appear to be distinct from each other (Kimura-Kuroda and Yasui, 1983) demonstrating the structural separation of specific activities on the viral E protein.

### 1.4.2 Oligonucleotide Mapping of JEV Isolates

RNA fingerprinting was used to examine the genetic makeup of JEV isolates. Some studies suggested that geographic boundaries limited migration of JEV and viruses isolated at approximately the same time were quite similar (Hori, 1986; Hori et al., 1986).

A panel of JEV strains were separated into six groups based on geographic and temporal boundaries. One of these groups contained the isolate Muar from Singapore, which had previously been shown to be serologically distinct from other JEV isolates (Kobayashi et al., 1984; Kobayashi et al., 1985).

### 1.4.3 JEV Strain Variation at the Amino Acid Level

Examination of nucleotide sequences is an excellent way for describing the genetic variation of viruses within particular geographical areas. However, sometimes the genetic variation seen between virus strains is not translated to the protein level and mutations are silent and in this case the structure of the virus is not affected. Therefore, examination of virus strain variation at the amino acid level is important to understand the true variation within a virus population (Holbrook and Barrett, 2002).

Analysis of the amino acid sequence of the E protein of 35 strains of JEV demonstrated that while the genotypes were conserved among the strains examined, some of the viruses that

were closely related genetically were less well related at the amino acid level (Mangada and Takegami, 1999). For example, antigenic examination of 12 Thai strains from genotype I and comparison with two Japanese and a single Chinese strain (GIII) demonstrated that the Thai strains have a definite antigenic difference compared to Nakayama and JaGAr01 strains (Ali and Igarashi, 1997).

The maximum genetic difference between JEV genotypes was found to be 10% and the maximum within genotypes was 6%, demonstrating that the JEV strains examined are closely related with the exception of the Muar virus (Chen et al., 1992; Chen et al., 1990; Williams et al., 2000). Therefore, based on the nucleotide and amino acid sequences of the E protein, it has been suggested that JEV isolates exist as a single serotype (Tsarev et al., 2000) .

However, cross-referencing genetic and antigenic data for JEV isolates is difficult as many of the older isolates have never been sequenced and many of the recent isolates have not been examined serologically.

## 1.5 Molecular Virology of JEV

### 1.5.1 Viron

JEV belongs to the JE serogroup of genus *Flavivirus*, family *Flaviviridae.* It is a small-enveloped virus with a single-stranded, positive-sense RNA genome (Zhang et al., 2009).

JEV has a genome structure, which is similar to that of other flaviviruses. The genome contains a single long ORF flanked by 5` and 3` nontranslated regions (NTRs) that are important for viral replication (Sumiyoshi et al., 1987).

The RNA genome lacks a poly (A) tail at its 3` (Sun et al., 2009). The ORF is translated into a large polyprotein that is co- or posttranslationally processed into three structural and seven NS proteins whose genes are arranged in the genome as follows: C- prM- E- NS1- NS2A- NS2B- NS3- NS4A- NS4B- NS5 (Chambers et al., 1990; Lindenbach, 2001; Venugopal, 1994). Schematic of JEV genome is shown in Figure 1-3.

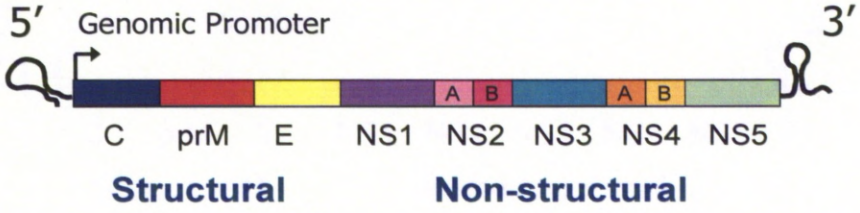


Figure 1-3. Schematic of Japanese encephalitis virus genome. Adapted from (http://expasy.org/viralzone/all_by_protein/24.html).

### 1.5.2 JEV Non Coding Regions (NCR)

The JEV genome contains 5' and 3' NCRs located at the extreme 5' and 3' ends of the virus genome (Markoff, 2003). The 5' and 3' NCR form secondary structures that are essential for replication and translation.

The 5' NCR is not conserved among members of the family *Flaviviridae* (Brinton and Dispoto, 1988; Pestova et al., 1998).

Mutations or deletions in the 3' NCR have been shown to reduce RNA replication (Elghonemy et al., 2005; Hanley et al., 2004; Khromykh et al., 2003; Lo et al., 2003). A DENV vaccine candidate has been proposed based on a mutant virus containing a 30 nucleotide deletion within the 3'NCR, which shows reduced replication in monkeys whilst still inducing a protective immune response (Hanley et al., 2004).

### 1.5.3 JEV Structural Proteins

JEV contains three structural proteins including the C, prM/M and E proteins. They are involved in various steps of the replication cycle and comprise the virus.

#### *C Protein*

The C protein is a small basic protein about 12 KD in size that binds viral RNA and is not highly conserved among flaviviruses (Khromykh and Westaway, 1996; Patkar et al., 2007).

#### *prM/M Protein*

The larger prM protein is the precursor to the small M protein (Amberg et al., 1994).

The role of the M protein has been demonstrated. A single mutation at M5 for JEV increased plaque size and infectivity in cell culture, but did not affect virulence in mice. Since this mutation was shown to inactivate the virus at low pH, it has been hypothesized that the M protein may function to stabilize the E protein in dimers at neutral pH from forming trimers (Maier et al., 2007).

The prM protein is associated with immature virus particles and is likely to protect the E protein from inactivation by the low pH during transport through the intracellular vesicles (Heinz and Allison, 2000). The prM protein also elicits a chaperone-like function preventing conformational changes in the E protein within the low pH secretory pathway (Guirakhoo et al., 1992). The prM protein is cleaved by furin or a furin-like protease after the virus leaves the trans-Golgi network, but before the release of the virus, to reveal the mature form of the virus (Murray et al., 1993; Stadler et al., 1997).

#### *E Protein*

The E protein is the major surface viral protein involved in tissue tropism, cell fusion, infection and immunogenicity as it elicits neutralizing antibodies (Zhang et al., 2011). Neutralizing antibodies against E protein are important for protective immunity and are involved in the clearance of viral infection.

The E protein lies parallel to the virion surface and forms an antiparallel dimer at physiological pH with a shift to a trimeric form at acidic pH (Heinz and Allison, 2000).

The X-ray crystallographic structure of the E protein for TBE, DEN-2 and WN viruses revealed some similarities including three domains; domain I, elongated dimerization, domain II containing the fusion peptide and an immunoglobulin-like domain III (Modis et al., 2003; Nybakken et al., 2006; Rey et al., 1995b; Zhang et al., 2004). The putative receptor binding domain III for mosquito-borne flaviviruses, including JE, YF and WN viruses, contain an RGD/RGE integrin binding recognition motif (van der Most et al., 1999). Figure 1-4 shows the X-ray crystallographic structure of the TBEV E protein.

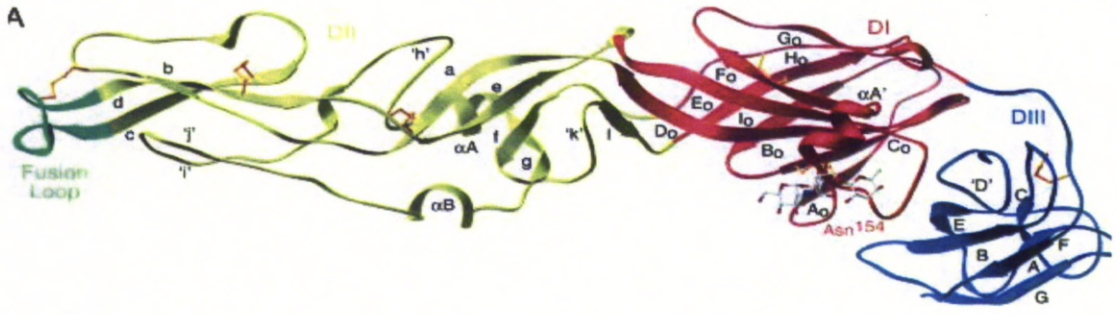


Figure 1-4. X-ray crystallographic structure of tick borne encephalitis virus envelope protein. The E protein is comprised of three domains: domain I is the central domain with 128 residues, domain II is the dimerisation domain with 171 residues and domain III is the contiguous stretch with 100 resides. Taken from (Kolaskar & Kulkarni-Kale, 1999).

Several regions of structural disparity are found between the TBEV structure and the JEV model. These regions of structural variation map to all three domains of the E protein, but the majority of differences are found in domains I and III (Rey et al., 1995).

A study of Korean isolates of JEV identified three amino acid changes among five isolates on the top surface of the domain III barrel, though none were predicted to have a significant effect on the antigenicity (Chung et al., 1996). However, the local conformation of a protein can be affected by changes in the amino acid architecture in other regions of the protein.

Comparison of JEV strains isolated in China, Japan, and India demonstrated that the viruses had a number of amino acid substitutions within the viral E protein when compared to the structure-based antigenic determinant predictions (Mangada and Takegami, 1999; Ni and Barrett, 1996; Vrati et al., 1999). However, few substitutions fell within predicted antigenic regions.

### 1.5.4 JEV NS Proteins

JEV synthesizes seven NS proteins inside virus-infected cells, including NS1, NS2A, NS2B, NS3, NS4A, NS5B and NS5. These proteins are necessary for virus replication.

#### ***NS1 Protein***

NS1 protein is highly conserved among flaviviruses. It has extensive identity and homology between different flaviviruses (Lindenbach and Rice, 1999). The NS1 protein interacts with other proteins necessary for replication, including the NS2A and NS4A proteins (Kummerer and Rice, 2002; Lindenbach and Rice, 1999).

Although the function of this protein has yet to be identified, the NS1 protein has been identified as essential for replication (Lindenbach and Rice, 1997).

The NS1 protein has been suggested to possess immunomodulatory capabilities. High levels of circulating NS1 protein has been linked to serious DEN infection, which may lead to potentially lethal infection (Alcon et al., 2002; Avirutnan et al., 2006; Libraty et al., 2002b; Macdonald et al., 2005; Young et al., 2000).

The role of secreted NS1 protein is unclear, but it is thought to be involved in the immune complex formation (Young et al., 2000), induction of autoreactive antibodies against extracellular matrix proteins (Chang et al., 2002), and enhanced viral production (Alcon-LePoder et al., 2005).

Antibodies against NS1 have no neutralizing activity *in vitro* but do induce protective immunity, probably by antibody dependent cellular cytotoxicity and elicit passive protection in mouse and monkey models (Gibson et al., 1988).

Intersetingly, the NS1 protein for WNV has been shown to inhibit complement activation, which may prevent complement-dependent lysis of infected cells (Chung et al., 2006). This is contrary to studies with DENV where NS1 appears to activate complement (Avirutnan et al., 2006), suggesting a novel function for WNV NS1.

## ***NS2A Protein***

The NS2A is a hydrophobic and small protein about 22 KD in size (Kummerer and Rice, 2002).

The NS2A protein is required for viral assembly and has been shown to contribute to the virulence of the virus in mice (Liu et al., 2003; Liu et al., 2006).

## ***NS2B Protein***

The NS2B protein is a hydrophobic protein that contains a 40 residue central region that is a cofactor of the NS3 serine protease and is necessary for the activation of the protease (Chambers et al., 1993; Leung et al., 2001; Mastrangelo et al., 2007).

Cleavage of the NS2B/NS3 complex is necessary for replication (Chambers et al., 1995). The NS2B protein also may be involved in the cleavage of the C protein (Amberg and Rice, 1999).

## ***NS3 Protein***

NS3 protein contains an N-terminal serine protease and C-terminal helicase, with nucleotide 5`-triphosphatase (NTPase) and 5`-terminal RNA triphosphatase (RTPase) activities (Mastrangelo et al., 2007). It is thought that the NTPase may be required for the helicase to unwind viral RNA and RTPase may be involved in the formation of the 5' cap (Brinton, 2002). The protease is responsible for the proteolytic cleavage of the N-terminal/C-terminal regions of the C protein, NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/NS4B and /NS4B/NS5 junctions (Nowak et al., 1989). Figure 1-5 illustrates X-ray crystallographic structure of DENV NS3 protein.

It has been demonstrated that NS3 protein is also involved in host cell apoptosis through the recruitment of caspase-8 (Ramanathan et al., 2006).

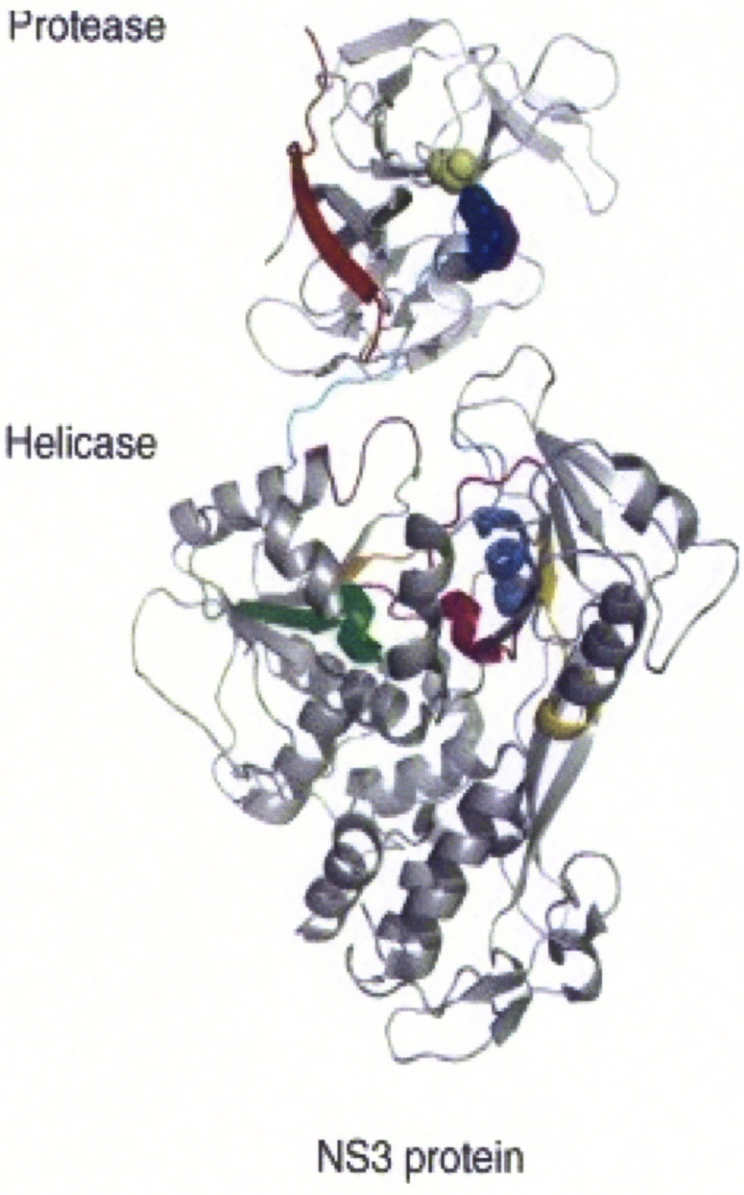


Figure 1-5. X-ray crystallographic structure of flaviviruses NS3 protein. NS3 protein is trifunctional, containing domains for protease, helicase and RNA triphosphatase. Taken from (Luo et al., 2008).

## *NS4A Protein*

The NS4A protein is a small hydrophobic protein, approximately 16 KD in size. It is involved in the replication complex.

Cleavage of NS4A protein may be regulated since two precursors, NS3/NS4A and NS4A/NS4B, are found within the cell (Chambers et al., 1990; Preugschat et al., 1990).

The NS4A interacts with the NS1 protein in an undetermined fashion possibly essential for replication (Lindenbach and Rice, 1999).

## *NS4B Protein*

The NS4B is a small hydrophobic protein of approximately 27 KD, which may participate in the replication cycle and immune evasion.

The NS4B protein shows only 35% amino acid identity between DEN, WN and YF viruses (Umareddy et al., 2006), however, members of the family *Flaviviridae* share common ER, cytoplasmic regions and transmembrane domains (Miller et al., 2006) that may indicate similarities in the function of this protein between viruses.

Mutations within the central hydrophobic region of the NS4B protein have been identified in several attenuated and passage-adapted flavivirus strains, including: chimeric WN/DEN-4 and DEN-2/DEN-4 viruses, passage adapted DEN-4 virus, JE vaccine strain, YF vaccine strain and hamster passage adapted YFV strain, and a WNV mouse attenuated strain (Blaney et al., 2003; Hahn et al., 1987; McArthur et al., 2003; Ni et al., 1995; Pletnev et al., 2002; Wang et al., 2003; Wicker et al., 2006).

## *NS5 Protein*

The NS5 protein is approximately 100 KD in size and the largest flaviviral protein. It is highly conserved among flaviviruses, containing a ethyltransferase activity in the N-terminus, which is utilized in 5' capping, and a 3' viral RNA-dependent RNA polymerase (RdRp) required for replication (Khromykh et al., 1999). The X-ray crystallographic structure of flavivirus NS5 protein is shown in Figure 1-6.

The NS5 protein has been shown to elicit immunomodulatory capabilities, specifically in interferon inhibition (Best et al., 2005; Lin et al., 2006).

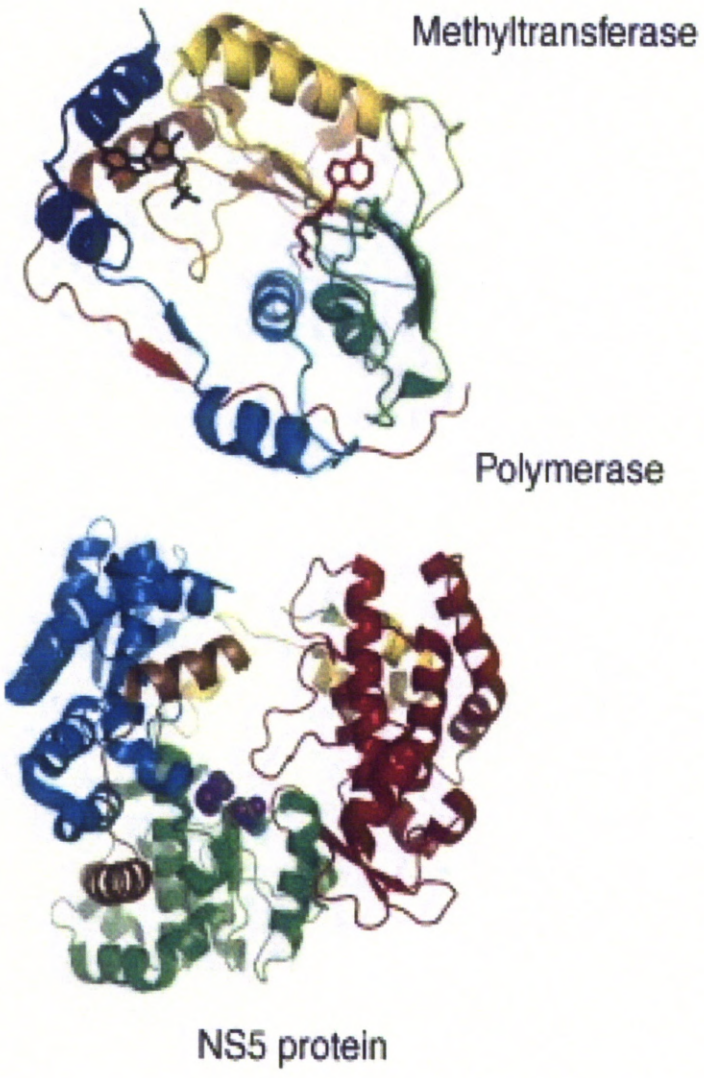


Figure 1-6. X-ray crystallographic structure of flavivirus NS5 protein. NS5 protein is RNA dependant RNA Polymerase and It is conserved among flaviviruses (10 conserved Cys residues). It contains a putative methyltransferase domain. Taken from (Zhou et al., 2007).

## 1.6 Molecular Evolution of JEV

JEV is one of the major encephalitic flaviviruses of public health importance. It belongs to the genus *Flavivirus*.

The *Flavivirus* genus in the family *Flaviviridae* comprises approximately 70 antigenically related members. Most members are arboviruses, transmitted by arthropod vectors, either ticks or mosquitoes, and usually replicate alternately in susceptible vertebrate and arthropod hosts. A few viruses have no known vectors. The most recent classification, as listed in the 7$^{th}$ Report of the International Committee on Taxonomy of Viruses (ICTV), has assigned members of the genus into species, with some members demoted to strains or serotypes. Thus there are now 27 mosquito-borne species, 12 tick-borne species, and 14 species with no known vector. The major criteria for determining the groupings of species, strains and serotypes within the genus are association and geographic incidence. The interpretation of the criteria for assigning a species level, and the definition of what constitutes a "strain" or "serotype" may be controversial in some instances, and indeed changes may be necessary as more information becomes available. Perhaps a more useful indication of relationships between flaviviruses can be obtained from phylogenetic studies which tend to agree with antigenic and vector/host relationships (Gould et al., 2001; Zanotto et al., 1996).

Molecular genetic classification of flavivurses has been attempted before. In all previous studies, fewer than one-third of the members, primarily mosquito-borne and tick-borne viruses, were used to create phylogenetic trees, which showed evolution of mosquito-borne and tick-borne viruses from the presumed ancestor (Zanotto et al., 1996).

### 1.6.1 Phylogenetic Relationships

The availability of complete or partial genome sequences for an increasing number of flaviviruses has provided the means for estimating phylogenetic relationships and the basis for hypotheses regarding their evolution (Drummond et al., 2003).

Phylogenetic analyses have been based on complete (Billoir et al., 2000; Cook and Holmes, 2006) or partial genome nucleotide sequences or predicted amino acid sequences of virus proteins. Most recent phylogenetic trees have been based on sequences of the NS3 and/or

NS5 genes (Billoir et al., 2000; Cook and Holmes, 2006; Gaunt et al., 2001). The basic segregation of tickborne viruses from mosquitoborne viruses was demonstrated in a dendrogram. Subsequently, phylograms confirmed this to be a primary evolutionary division and suggested that flaviviruses have evolved by divergent mutational change (Blok et al., 1992). Phylogenetic studies on E gene sequences revealed a difference in the rate of mutation with the mosquitoborne group having nearly twice that of the tickborne group (Zanotto et al., 1995).

### 1.6.1.1 Building Phylogenetic Trees

A phylogenetic tree is a simple way to show evolutionary relationships between taxa which may be unrooted or rooted. Rooted trees include the position of the last common ancestor of each tree member.

Principles of phylogenetic tree building were discussed in detail by (Felsenstein, 1988, 2001; Goldman, 1993; Jukes, 1969; Lanave et al., 1984; Posada and Crandall, 1998) and are briefly mentioned here.

There are two types of tables that show the relation between taxa at either the percentage of matches (similarity table) or percentage of differences (distance table). The table will be converted into a matrix to be used to construct a phylogenetic tree.

There are several software packages for phylogenetic analysis such as PAUP (phylogenetic analysis using parsimony) and PHYLIP (phylogenetic inference package). There are different methods of phylogenetic analysis including distance matrix, maximum parsimony (MP) and maximum likelihood (ML).

However, there are some limitations of phylogenetic algorithms such as incorrect sequence alignment, failure to account for variation of evolution rates at different sites within a sequence, and failure to account for sequences evolving at different rates in different taxa which will generate incorrect trees. Therefore, the problems can be addressed in several ways. The first is to make sure that sequence alignments are robust. Caution should be exercised in particular if a newly built tree disagrees with others generated through the analysis of different genes or proteins.

Reliability of the phylogenetic trees could be determined through bootstrapping in which data are randomly sampled from any position within a multiple sequence alignment, and are built into new artificial alignments, which are then tested by tree building. The trees built by bootstrapping should always match the original tree, and this would be defined as "100% bootstrap support".

### 1.6.2 Evolutionary Relationships

Theories of the evolution of flaviviruses must fit constraints imposed by (a) their observable genetic relatedness, (b) the ecology of the individual virus species in nature (e.g., vector and vertebrate hosts), and (c) the geographic distribution.

The conclusion that vectorborne flaviviruses arose in Africa is supported by the observation that all the tick- and mosquitoborne viruses that diverge at the lower nodes of the flavivirus tree are predominantly Old World viruses (Gaunt et al., 2001).

The hypothesis that mosquitoborne viruses arose from tickborne viruses or that the two clusters share a common ancestor is supported by the findings that viruses which are usually associated with mosquitoes, Koutango, Saboya, WN, and YF viruses, have been repeatedly isolated from ticks too (Billoir et al., 2000).

Differences in the speed of evolution between tick- and mosquitoborne viruses are mirrored at the amino acid sequence level. Adaptive substitution mutations in tickborne virus proteins are typically conservative, whereas those observed in mosquitoborne virus proteins are more likely nonconservative (Lobigs et al., 1990). The rate of mutation at the amino acid level between tick- and mosquitoborne viruses supports the concept of a slower evolutionary rate in the tickborne viruses (Zanotto et al., 1996).

JEV is believed to be one of the most recently diverged members of the family *Flaviviridae*, and most JEV isolates are estimated to have evolved over the last 130 years (Zanotto et al., 1996).

### 1.6.2.1 Evolutionary Analysis

There is no doubt that evolution and statistics are two common subjects that pervade the modern analysis of molecular sequence variation. Questions related to molecular sequences are statistical in nature and should be framed in terms of parameter estimation and hypothesis testing.

Bayesian evolutionary analysis by sampling trees (BEAST) software package is an ambitious attempt providing a general framework for both parameter estimation and hypothesis testing of evolutionary models from molecular sequence data (Drummond & Rambaut 2007). It uses a Bayesian statistical framework and thus provides a role for prior knowledge in combination with the information provided by data.

BEAST is a cross-platform program for bayesian markov chain Monte Carlo (MCMC) analysis of molecular sequences. It is oriented towards rooted, time-measured phylogenies inferred using strict or relaxed molecular clock models. It is intended both as a method of reconstructing phylogenies and as a framework for testing evolutionary hypotheses without conditioning on a single tree topology.

BEAST is a powerful and flexible evolutionary analysis package for molecular sequence variation as it provides considerable flexibility in the specification of an evolutionay model.

Substitution models describe the process of one nucleotide or amino acid being substituted for another. Transitions are interchanges of purines (A ↔ G) or pyrimidines (C ↔ T). While, transversions are interchanges of purine for pyrimidine bases. Figure 1-7 illustrates the possibility of transitions and transversions. However, some sites such as initiation codons may not be free to vary at all (Akmaev et al., 2000).

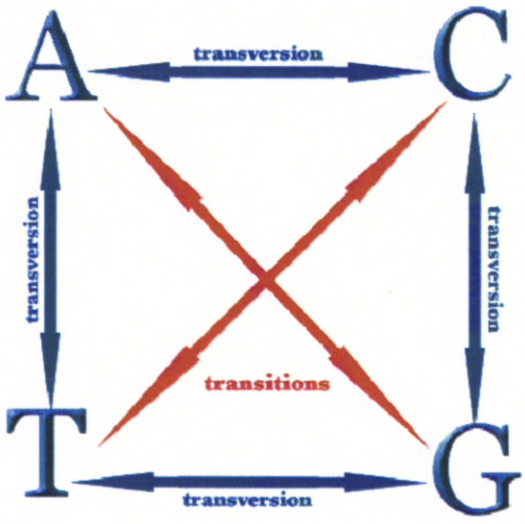


Figure 1-7. The possibility of transitions (red arrows) and transversions (blue arrows).

## 1.7 JE Infection and Disease in Humans

### 1.7.1 Incidence of JEV Infection and Disease Association

The incidence of JEV infection and encephalitis varies by country due to many factors, including the amount of virus circulating, people's exposure to the virus, the country's policy for vaccination against JE and underreporting of the disease. In endemic countries, JE is an illness of children under 10 years of age (Thisyakorn and Nimmannitya, 1985; Thongcharoen, 1985, 1989).

There is not much disease in infants; this is a reflection of maternal transfer of antibody that lasts between 8 and 12 months of age and host exposure to the vector. Adult immune naives are represented by travellers, tourists and military soldiers, are at significant risk of JEV infection and encephalitis when travelling to JE endemic areas (Ketel and Ognibene, 1971; Tsai, 2000).

### 1.7.2 Risk Factors for JEV Infection and Disease

Human JEV infection could be asymptomatic with antibody seroconversion, could induce a subclinical or febrile illness, or it could target the centeral nervous system (CNS) causing encephalitis as well as meningitis (Hosokawa et al., 2007).

People living in endemic rural areas of Asia are exposed to JEV during childhood, but only 1 in 300 develops disease (Vaughn and Hoke, 1992). Generally, the ratio of symptomatic to a symptomatic disease is estimated to be 1:50 - 1:300 (Libraty et al., 2002a).

The factors determining which of all the infected humans with JEV develops disease could include viral factors such as strain differences, site of entry, and inoculating dose, as well as host factors such as living in endemic areas for JE, age, genetic make-up, general health and pre-existing immunity, lack of JE-specific antibody from natural infection or vaccine, and behaviour that increases exposure to the mosquito vector such as night exposure or lack of bed nets and mosquito repellents.

## Exposure to Infected Mosquito Vector

Human behaviour that affects exposure to infected mosquitoes may play an important role in getting JEV infection. Humans become infected during the bite of an infected mosquito. Incidence of JE in young children (<3 years old) is less than in older children because they play outside less after dusk (Hoke et al., 1992; Huang, 1982).

The risk of JE increases as the number of vector mosquitoes increases. Because the rice paddy-breeding *Culex* mosquitoes are unavoidable, the majority of the population in rural Asia has been infected with the virus by early adulthood. The expansion of JEV-endemic area depends on irrigated rice field and pig farming. These environments that are suitable for the infectious cycle of JEV exist widely in Asia (Oya and Kurane, 2007).

## Virus Strain Differences

It has been suggested that strain-specific differences in neurovirulence may determine the clinical presentation as it has been observed that JEV isolates from northern Thailand, where the disease is epidemic, are of a different genotype than isolates from southern Thailand, where the disease is endemic (Chen et al., 1990). However, in Vietnam, both epidemic and endemic patterns are caused by a single genotype (Huong et al., 1993).

Wild-type isolates of JEV with differing phenotypes in terms of neurovirulence and neuroinvasiveness in mice do occur (Hasegawa et al., 1992; Huang and Wong, 1963; Ni and

Barrett, 1996). It is unknown whether the same could be true for humans (McMinn, 1997). Though, more subtle strain differences may be important as the reported genotyping is based on a limited portion of the genome (Solomon and Vaughn, 2002).

## Protective Effect of Anti-flavivirus Antibodies

The most important risk factor for developing severe encephalitis is the lack of flavivirus antibody prior to JE infection (Burke et al., 1985a; Edelman et al., 1975; Sather and Hammon, 1970). Children with pre-existing flavivirus antibody (most often presumed to be DENV in Southeast Asia) have milder JE disease which may be due either to the cross-protective property of flavivirus antibody that modifies disease severity, or to a marker that results in cross-protective T-cell responses.

Some JE endemic areas are also endemic for other flaviviruses, such as DENV. However, prior anti-flavivirus antibodies do not entirely protect against JEV infection but they may make infections less severe (Grossman et al., 1973).

It has been observed that children younger than 10 years of age are more likely to die or to have severe sequelae than older children and young adults (Schneider et al., 1974). Older patients with JE have a secondary anti-flavivirus antibody response suggesting that previous infection with a different flavivirus may give some protection (Endy and Nisalak, 2002).

The reason by which older patients are protected is thought to be due to cross-protective immunity from prior DENV infection (Edelman et al., 1975; Grossman et al., 1973; Libraty et al., 2000).

## Crossing the Blood-Brain Barrier (BBB)

Humans get JEV infection during the bite of an infected mosquito. Following inoculation, JEV is thought to replicate in the skin before being transported by Langerhans' dendritic cells to local lymph nodes as has been observed in the experimental intradermal (i.d.) infection of BALB/c mice with WNV and in volunteers receiving candidate live-attenuated DENV vaccines (Johnston et al., 2000; Wu et al., 2000).

It is not well known how neurotropic flaviviruses enter the CNS. The two postulated routes are through the olfactory mucosa where there is no BBB, or via hematogenous spread (Rapoport, 1976).

Intranasal inoculation (i.n.) is an effective means of experimentally inoculating rhesus monkeys with JEV and virus replication was widespread in the CNS and not always identified in the olfactory bulb suggesting hematogenous spread (Myint et al., 1999; Raengsakulrach et al., 1999).

However, i.d. inoculation in mice, showed that JEV establishes infection in the regional lymph nodes, then spreads via the blood to other organs such as liver, heart, and kidney, before crossing into the brain. A close relationship was shown between virus multiplication in the periphery and cerebral involvement (Huang and Wong, 1963). It is thought that JEV also replicates in the periphery in humans before crossing the BBB as in humans, JEV was isolated from the liver of one fatal case, and the damage in the lungs, myocardium, liver, kidney, and reticuloendothelial system has been demonstrated histologically (Haymaker and Sabin, 1947; Miyake, 1964; Mukherhi and Bisws, 1976; Zimmerman, 1946).

The actual means mechanism by which JEV and WNV cross the BBB have been studied. In theory, virus could cross either by passive transport across the endothelium, by replication in the endothelial cells, or within infected inflammatory cells that enter the brain parenchyma. It has been shown that virus replication in endothelial cells is important for WNV but not for JEV. *In vitro*, endothelial cells are readily infected with WNV causing cytopathic effects (CPE), and electron microscopic examination reveals antigenic localization (Dropulie and Masters, 1990). In contrast, ultrastructural examination of the mice brains injected with JEV shows that the virus undergoes endocytosis and transportation across endothelial cells and pericytes without replication (Liou and Hsu, 1998).

In some trials for understanding how WNV as one of the encephalitic viruses crosses the BBB. Experimental inoculation of WNV in mice showed that toll-like receptor 3 (TLR3) binding to the viral double-stranded RNA results in the generation of antiviral and immunomodulatory cytokines. A reasonable prediction is that targeted deletion of TLR3 would lead to increased peripheral viral infection with enhanced viremia. The higher level of

virus in the blood would lead to greater spread across the BBB (Diamond and Klein, 2004; Wang et al., 2004). Figure 1-8 illustrates a model of how WNV may cross the BBB.

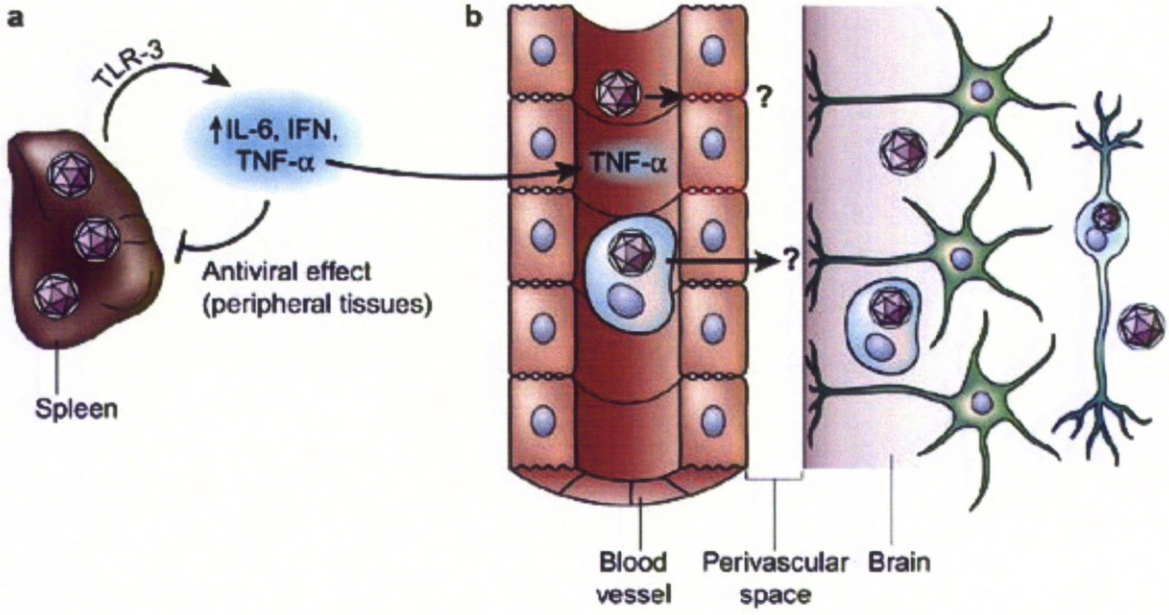


Figure 1-8. WNV crossing the BBB. (a) Infection of macrophages or dendritic cells by WNV in peripheral lymphoid tissue induces TLR-3-dependent secretion of antiviral and immunomodulatory cytokines which inhibit WNV infection in peripheral tissues. TLR-3-dependent induction of TNF-α, however, also facilitates WNV penetration across the BBB. (b) TNF-α may alter endothelial cells or endothelial cell tight junctions so that WNV or WNV−infected leukocytes traffic across the perivascular space and glial cell foot processes to infect neurons. Taken from (Diamond and Klein, 2004).

It has been shown that concomitant conditions that result in a disruption of the BBB, allowing JEV to cross into and replicate in the CNS may play a role in the pathogenesis of JE encephalitis. For example, cerebral cysticercosis may be a poor prognostic indicator for JE (Desai et al., 1997).

## Structural Abnormalities of the Brain

JEV affects both adults as well as children when it reaches a new geographic area for the first time. However, adults over 60 years of age appear to be more likely to have severe disease (Kitaoka, 1972). In Japan and Korea where widespread vaccination and changed socioeconomic circumstances led to reduction in the number of JE cases, the disease is now seen mostly in the elderly (Kono and Kim, 1969).

Possible reasons for the increased attack rate and severity in the elderly include structural abnormalities that facilitate viral transfer across the BBB, general poor health, and impaired immunity (Solomon and Vaughn, 2002).

A high incidence of cysticercosis has been noted among fatal JE cases (Desai et al., 1997; Liu et al., 1957; Shankar et al., 1983). There was a strong association between JE and neurocysticercosis, and, thus, this coinfection was more than a chance occurrence (Handique et al., 2008).

In Saipan, three out of ten JE patients had a structural brain abnormality preceding JE (one with a craniotomy, one with severe trauma, and one with a cerebrovascular accident) (Paul et al., 1993). Even mild head trauma during the transient viremic phase could facilitate viral entry into the CNS (Shiraki, 1970).

## Impaired Immunity with Age

Humoral and cellular immunity play an important role in protection against JEV infection. It has been shown experimentally in animal models that the cellular immune response may contribute to the prevention of disease during acute infection by restricting virus replication before the CNS is invaded (Solomon and Vaughn, 2002).

The susceptibility of athymic nude mice to experimental JEV infection has increased (Yu, 1985), and transfer of spleen cells from mice immunized with live-attenuated virus conveys immunity to infection (Jia et al., 1992). Spider monkeys, which are normally unaffected by intracerebrally inoculated JEV develop rapidly progressive encephalitis when T-cell function has been impaired by cyclophosphamide (Nathanson and Cole, 1970).

However, there is little direct evidence to suggest that an impaired immune response contributes to JE in humans. In Japan and Korea, the increased incidence of JE in elderly people, who presumably were previously immune because of early exposure to the virus, may indicate a waning of immunity. In humans infected with SLEV impairment of T-cell function by human immunodeficiency virus (HIV) appears to increase the risk of developing encephalitis (Solomon and Vaughn, 2002).

## JE and Travellers

Overall, travellers to Asia are at extremely low risk to acquire JE. However, the risk in the individual traveller may be highly variable, depending on area being visited, season, duration of travel and activities of the person (ACIP, 1993). Although considered to be rare in short-term tourists, JE has been reported after brief stays in Bali in at least two cases (Macdonald et al., 1989; Wittesjo et al., 1995).

Therefore, a JE vaccine should be considered for travellers visiting JE endemic or epidemic areas regardless of the duration of their visit (Mayer and Neilson, 2010).

### 1.7.3 Clinical Features of JE

Although epidemics of encephalitis occurred in Japan from the 1870s onwards, clinical features of JE were not described until World War I when American service personnel were exposed to the virus (Solomon and Vaughn, 2002).

Patients with JE present after a few days of a nonspecific febrile illness, which include headache, nausea, cough, vomiting, diarrhea, and rigors (Solomon et al., 2000). In older children and adult patients, abnormal behaviour may be the only clinical feature, resulting in an initial diagnosis of mental illness. During the Korean conflict some American servicemen with JE were initially diagnosed as having 'war neurosis' (Lincoln and Sivertson, 1952). Some patients make a rapid spontaneous recovery as they have aseptic meningitis only with no encephalopathic features (Chan and Loh, 1966).

The classical feautures of JE include a 'Parkinsonian' syndrome of dull flat 'mask-like' faces with wide unblinking eyes, tremor, generalized hypertonia, cougwheel rigidity and other

movement abnormalities. In addition, there may be upper motor neuron signs (increased tone, brisk reflexes, extensor plantars), cerebellar signs and cranial nerve palsies. Relative bradycardia has been noted in some patients with JE (Sabin, 1947) and hypertension has occasionally been reported (Poneprasert, 1989).

Convulsions have been reported in up to 85% of JE infected children (Kumar et al., 1990; Schneider et al., 1974) and 10% of adults (Dickerson et al., 1952; Poneprasert, 1989). Some form of JE motor paralysis as well as the increased tone, brisk reflexes, and extensor planter reflexes are suggestive of upper motor neuron paralysis, 5%-20% of comatose patients have flaccid weakness with reduced or absent reflexes that is characteristic of lower motor neuron lesions (Dickerson et al., 1952; Kumar et al., 1991; Sabin, 1947; Schneider et al., 1974).

As well as comatose patients with flaccid weakness, there is a subgroup of fully conscious JEV infected patients with a polio-like acute flaccid paralysis (Solomon et al., 1998).

Following a short febrile illness there is a rapid onset of flaccid paralysis in one or more limbs, despite a normal level of consciousness. Weakness occurs more frequently in the legs than in the arms, and is usually asymmetrical. Occasionally it affects only the respiratory muscles. Thirty percent of such patients subsequently develop encephalitis, with a reduced level of consciousness, and upper motor neuron signs, but in the majority acute flaccid paralysis is the only feature. At follow-up there may be persistent weakness and marked wasting in the affected limbs (Solomon and Vaughn, 2002).

### 1.7.4 Outcome in JE

JE case-fatality rates range from 0% to 55% with most reports around 30% (Benenson et al., 1975; Burke et al., 1985a; Grossman et al., 1973; Hoke et al., 1991; Kono and Kim, 1969; Kumar et al., 1990; Paul et al., 1993; Sabin, 1947; Schneider et al., 1974; Sunnara and Touch, 1995). With better hospital facilities, it has been observed that mortality rates may be lower with a concomitant increase in the proportion of patients with sequelae (Solomon, 1997).

Twenty percent of patients have severe cognitive and language impairment (most with concurrent motor impairment), and 20% have further convulsions (Huy et al., 1994; Kumar et al., 1993).

Patients with an apparently good recovery may have more subtle sequelae such as learning difficulties and behavioural problems (Kumar et al., 1993). In most patients JE is an acute infection, however, chronic progressive encephalitis and relapse, possibly caused by persistence of the virus in the CNS, have been reported in few patients (Pradhan et al., 2000; Ravi et al., 1993; Sharma et al., 1991).

### 1.7.5 Prognosis in JE

Increasing age is a risk factor for severe disease in many arboviral encephalitis, however, in JE younger children have a worse prognosis than older children and young adults. The eldery are also more likely to experience severe disease. The mechanism by which older children and adults are protected includes a degree of cross-protective immunity from prior DENV infection. In support of this is the observation that positive virus isolation from the CSF, and a failure of immunoglobulin (Ig) M and IgG production in the CSF and serum are associated with a fatal outcome (Burke et al., 1985a).

A poor JE prognosis includes deep coma (Burke et al., 1985a; Kumar et al., 1990; Solomon et al., 2000), abnormalities of respiratory pattern, increased or decreased tone, extensor or flexor posturing and other signs of brainstem damage (Kumar et al., 1990; Solomon et al., 2002), frequency of seizures, higher admission temperature, absent abdominal reflexes, hyponatremia, and low serum iron (Dapeng, 1995; Kumar et al., 1990; Libraty et al., 2000; Misra et al., 1998; Solomon et al., 2002; Solomon et al., 1996) and signs compatible with brainstem herniation through the foramen magnum were also associated with a poor outcome (Solomon et al., 2002).

JE-specific antibody that results from infection is life-long in duration. Seroprevelance studies indicate that in areas where JE circulates, there is an age-dependent increase in JE antibody and most adults have been exposed to and infected by JEV (Solomon and Vaughn, 2002).

### 1.7.6 Treatment of JEV

Despite the significant disease burden caused by JEV, no specific antiviral therapy is currently licensed for treatment (Ghosha et al., 2008). JEV is treated symptomatically.

However, some compounds have shown some anti viral acttivity against JEV *in vitro* or in animal models; such as isoquinolines, anti-JEV MAbs, nucleoside analogs, the lymphocyte modulator Concanavalin A, nitric oxide and ribavirin (Kimura-Kuroda and Yasui, 1988).

Salicylates and nonsteroidal anti-inflammatory drugs (NSAID) were shown to inhibit the *in vitro* replication of JEV, and prevent apoptosis of infected cells (Chen et al., 2002).

Corticosteroids were given for JE, however in placebo-controlled trials there were no benefit associated with the administration of corticosteroids or IFN-α (Hoke et al., 1992; Solomon et al., 2003a). IFN-α, a glycoprotein cytokine that is produced naturally in response to viral infections, including JEV (Burke and Morrill, 1987), has been the most promising antiviral aganist JEV infection. In tissue culture, recombinant interferon is effective against JEV and other arboviruses including WNV (Anderson and Rahal, 2002).

Since several years, the use of IVIG has increased. IVIG is a blood product administered intravenously. It contains the pooled IgG extracted from the plasma of over one thousand blood donors. IVIG's effects last between 2 weeks and 3 months.

IVIG has been used recently to treat flavivirus encephalitis. There has been a case report of, IVIG which has been shown which appeared to be of benefit when administered with symptomatic treatment for JEV. In the case report, the patient's clinical signs has improved rapidly with almost complete recovery (Caramello et al., 2006). It has been sugessted that such antibody- containing immunoglobulin may provide a specific and effective treatment for serious cases of flaviviruses such as WNV infections (Shimoniz *et al.*, 2001).

### 1.7.7 Diagnosis of JE

JE diagnosis is not easy, especially in areas where two or more related flaviviruses co-circulate. For example in Southeast Asia, all four DENV serotypes circulate and in southern India, in addition to DENV, WNV also circulates. In northern Australia, both KUNV and MVEV circulate (Endy and Nisalak, 2002).

Therefore, the ideal assay for diagnosis of acute JE infection and encephalitis should be highly sensitive and specific. It should have the ability to distinguish JE from other flaviviruses.

The co-circulation of similar flaviviruses with a high-degree of cross-reactive human antibody creates a challenge for serologic assays to detect JE and distinguish it from other flaviviruses (Wisseman et al., 1966). For example, a person with previous exposure to DENV who becomes infected with JE will have an increase in antibody to both JE as well as the four serotypes of DENV as detected by antibody-based assays such as HI or plaque reduction neutralization titer (PRNT).

Currently the diagnosis of JE infection is based on serologic, viral isolation, molecular, and immunocytochemistry assays.

#### Serology

#### *(A) Haemagglutination Inhibition (HI)*

Although HI was the first assay to measure human antibody specific for the arthropod-borne viruses including JE (Clarke and Casals, 1958) it is still a fundamental tool in arboviral and JE diagnostics.

Hemagglutination assay (HA) is the precursor to the HI assay, which showed that arboviruses, in particular JEV, were able to agglutinate certain types of erythrocytes (Sabin, 1951; Sabin and Buescher, 1950).

The HA assay allowed quantification and standardization of JEV antigen produced from a variety of sources including the most common, suckling mouse brain. The finding that

arbovirus-specific antibody was able to inhibit HA of erythrocytes created a relatively simple and inexpensive HI assay which to measure JE-specific antibody (Clarke and Casals, 1958).

The main advantage of the HI assay is that it can be performed with minimal laboratory equipment, reagents and expense. However, it could not discriminate adequately between the closely related flaviviruses such as DENV and WNV. This produces results that are difficult to interpret in countries where these viruses co-circulate. Despite these limitations, the HI assay is a powerful technique that is still a standard assay for seroprevelance studies as well as in the diagnosis of acute primary and secondary DENV infections (Endy and Nisalak, 2002).

### *(B) IgM and IgG Enzyme Immunoassay*

ELISA is widely used in the diagnosis of many viruses. Detection of IgM during acute JEV infection provides a highly specific antibody based assay without the problems of cross-reactivity that IgG antibody displays for other flaviviruses. This was demonstrated for group B arbovirus infections. For example, in DENV vaccinated gibbons challenged with JEV and in patients hospitalized with JE infection (Edelman et al., 1973; Edelman and Pariyanonda, 1973; Scott et al., 1972).

On the basis that IgM does not cross the BBB, its detection in CSF was performed using an antibody capture radioimmunoassay, which was modified and simplified to an ELISA format using an IgM isotype specific antibody capture immunoassay (Burke and Nisalak, 1982; Burke et al., 1986; Burke et al., 1982; Burke et al., 1985b). The IgM isotype specific antibody capture immunoassay demonstrated a sensitivity of 73% and a specificity of 100% when used during a field trial (Burke et al., 1986).

ELISA as well as a dot enzyme immunoassay has also been used to detect JE IgM in swine sera with high sensitivity and specificity (Burke et al., 1985c; Cardosa et al., 1993).

Antibody-capture ELISA approach yielded a sensitive and specific method to distinguish acute JE from acute DENV infections (Bundo and Igrashi, 1985).

An antidengue isotype capture ELISA has been developed to characterize DENV infections in regions where DENV and JEV co-circulate (Innis et al., 1989). This assay provided a convenient format to distinguish acute DEN from acute JEV infections. It used an anti-DENV isotype-capture enzyme immunoassay to measure the proportion of Ig isotype reactive with DENV of JE antigen.

### *(C) Plaque Reduction Neutralization Titer Assay (PRNT)*

Arbovirus plaques were detected using an agar overlay stained with neutral red (Henderson and Taylor, 1959). This technique allowed an easy *in vitro* assay to directly detect and measure the infectivity of a virus preparation or stock as plaque forming units. Its application to JEV awaited the finding that JEV produces CPE (plaques) in certain cell lines such as in cultured baby hamster kidney (BHK) cells and in chick embryonic fibroblast monolayer cultures (Diercks and Hammon, 1958; Kissling, 1957; Porterfield, 1959).

PRNT for JEV was developed using cultured chick embryonic fibroblasts and demonstrated a good correlation to HI antibody titers (Yoshioka et al., 1965). This technique was subsequently adapted to the green monkey kidney cell line, Vero cells, using a constant-virus decreasing serum technique and was more sensitive than HI titers (Yoshioka et al., 1966).

PRNT protocols are being used to measure JE neutralizing antibody using a variety of cell lines, including LLC-$MK_2$ (Kidney, Rhesus monkey, *Macaca mulatta*), Vero and BHK21 cells. The endpoint is the titer of antibody that will reduce a certain percentage of viral plaques such as a 50%, 70%, or 90% plaque reduction ($PRNT_{50}$, $PRNT_{70}$, $PRNT_{90}$, respectively) compared to control cultures. Higher PRNT values increase the specificity but decrease the sensitivity of the assay, therefore a $PRNT_{50}$ is the most commonly used endpoint (Endy & Nisalak, 2002).

A JE antibody titer as measured by the PRNT assay is interpreted in a number of ways. In a patient with suspected acute JE infection, a titer increasing by fourfold suggests an acute infection. A titer of 1:10 or more suggests past exposure or vaccination, and protective immunity.

### *(D) Western Blot*

Western blot is a technique that utilizes the characteristic of proteins to travel through a polyacrylamide gel, and to transfer onto nitrocellulose sheets when an electrical current is passed through the gel (Towbin et al., 1979).

Separation of proteins occurs as they travel through a gel at different speeds depending on their molecular weight and charge. The result, when applied to proteins derived from JE, is a spread of different structural and NS protein components across a gel, predominantly E, NS1, and pre-M. These proteins are then transferred onto nitrocellulose paper by an electrical current applied horizontally to the gel. Human sera can then be applied to the nitrocellulose strip and human antibody specific to JE protein will bind; an anti-human antibody conjugate system is then used to detect binding of the human antibody. The result is a band showing the presence of antibody to those proteins (Endy and Nisalak, 2002).

Western blot has limited diagnostic utility as JEV and DENV proteins are highly cross-reactive and this assay is unable to distinguish the two viral infections.

## Virus Isolation

JEV Isolation during an acute infection is extremely difficult, partly due to the high levels of neutralizing antibody that exist at the time that clinical symptoms occur.

Some studies illustrate the difficulties in isolating virus from nonfatal cases of JE. In one series of 101 patients with JE infection of the CNS, no virus was isolated when the geometric mean titer of neutralizing antibody exceeded 1:500 at the onset of clinical symptoms (Kimoto et al., 1968). In a similar series of patients in Thailand, among 49 cases of JE infection of the CNS, no plasma specimens or CSF yielded virus in the nonfatal cases. Only in 5 of the 15 fatal cases of JE was there virus isolation from the CSF and 7 of 15 had virus isolation from brain tissue (Leake et al., 1986).

JEV isolation from nonfatal cases can be performed using a variety of methods, the classic being intracranial inoculation (i.c.) of clinical specimen in suckling mice. Normally, 1- to 2-day-old suckling mice (*Mus musculus*) are inoculated i.c. with 0.02 ml of a suspension of

clinical material. Mice are observed twice a day for the first signs of encephalitis (failure to eat as evidenced by lack of milk in the stomach, colour change, wasting, runting, or tremors), which occur approximately 3-10 days after inoculation. Confirmation of JEV is performed by staining the brain with either a polyclonal or monoclonal antibody specific for JE conjugated with a fluorescent tag (Endy and Nisalak, 2002).

A more direct method of JEV isolation could be carried out by direct inoculation and isolation in a variety of mammalian and insect cell lines such as Vero and LLC-$MK_2$ cells from monkey kidneys, BHK21 from hamsters, pig kidney epithelial cells, AP61 from *Ae. pseudoscutellaris*, C6/36 from *Ae. Alopictus* (Fu and Zhang, 1996; Igarashi et al., 1981; Igarashi et al., 1973).

Other method for JEV isolation is inoculation of patient sera into mosquitoes to amplify and propagate the virus. A variety of mosquito vectors has been used including *Toxorhynchites splendens, Ae. Albopictus, or Ae. Aegypti* (Rosen and Gubler, 1974). *Toxorhynchites* mosquitoes have the advantage of being larger and easier to inoculate with human.

Viral isolation in fatal human cases of JE infection is best performed on tissue or autopsy specimens of brain tissue (George et al., 1987b).

## Molecular: Polymerase Chain Reaction (PCR)

PCR has been used with success for diagnosis of flaviviruses. It is a molecular technique based on the ability to amplify small amounts of RNA or DNA to detectable levels using molecular primers, a polymerase enzyme and a thermocycler.

The limitations of PCR are similar to the limitations with the isolation of virus. Viremia and detectable viral RNA in sera or CSF is found infrequently at the time of clinical illness.

PCR may have higher yield when performed using brain biopsy specimens although this has not been demonstrated in clinical studies (Endy and Nisalak, 2002).

## Immunocytochemistry

Immunohistochemistry is the staining of the specimens for the presence of specific proteins and as it applies to JE. JE antigen staining is a powerful technique to diagnose fatal cases of JE when serology or viral isolates are not available.

A number of techniques have been used to detect arbovirus antigen in tissue specimens to include both direct and indirect fluorescent antibody staining, and enzyme conjugates using peroxidise and alkaline phosphatase conjugates (Endy and Nisalak, 2002).

Immunohistochemistry is very useful in diagnosis of JE fatal cases when sera or CSF are not available. In one series 62% of fatal cases of JE were diagnosed by immuohistochemical staining for JE antigen in brain tissue (Zhi-shang et al., 1988).

## 1.8 Immune Responses to JEV

Understanding the immune responses which contribute to the prevention of and the recovery from JE is important to the development of new types of vaccines and specific treatment. Also analysis of immune responses to JEV as a model will provide information useful to the understanding of immune responses to other encephalitic viruses.

### 1.8.1 Components of Protective Immunity to JEV

It has been found that neutralizing antibodies play a key role in protection, while cytotoxic T lymphocytes (CTLs) play a key role in the recovery (Kurane, 2002).

Pre-existing neutralizing antibodies are important for protection from JEV infection. It was demonstrated that passive transfer of neutralizing antibodies before virus inoculation protected mice from lethal challenge (Kimura-Kuroda and Yasui, 1988b; Mason et al., 1989; Oya, 1966, 1988) and also partial protection was achieved when antibody was transferred passively after infection.

JEV infection induces antibodies to both NS and structural proteins. Antibodies to NS1 also protected mice from lethal flavivirus infection when transferred passively (Henchal et al., 1988; Schlessinger et al., 1985).

Antibodies to NS1 do not neutralize viruses; although complement –mediated cell lysis and antibody-dependent cell-mediated cytotoxicity were attributed to protective activity, the mechanism of protection by anti-NS1 antibodies is incompletely understood. It has been observed that monoclonal antibody to DENV preM protein protected mice from lethal infection (Kaufman et al., 1989); it is possible, therefore, that antibodies to JEV NS1 and preM may also have a protective effect against lethal infection.

The role of CTLs in the recovery from JEV infection has not been well established. However, it has been reported that passive transfer of virus-specific CTLs also protected mice from lethal challenge by JEV (Murali-Krishna et al., 1996).

### 1.8.2 JEV Proteins that Induce Protective Immunity

It has been demonstrated that the E protein induces protective immunity; this has been detected by using extracellular particles, and recombinant vaccinia virus and DNA vaccines containing the E gene (Chen et al., 1999; Konishi et al., 1991; Konishi et al., 1998b).

Although neutralizing antibodies that recognize the E protein play a major role in protection from JE infection, it has been reported that a DNA vaccine containing the NS1 gene induced protective immunity (Lin et al., 1998); however, the levels of protection elicited by NS1 were very low compared to studies by other investigators (Chen et al., 1999).

Other viral proteins do not elicit protective immunity. Thus, it is likely that the neutralizing antibodies to the E protein, non-neutralizing antibody to NS1 protein and specific T lymphocytes are involved in protective immunity against JEV (Kurane, 2002).

### 1.8.3 Immune Responses to JEV in Humans and Mouse Models

Human immune responses to JEV have been analyzed using specimens from JE patients, and from those who recovered from JE. High levels of neutralizing antibodies directed to E protein are induced by infection with JEV (Konishi et al., 1995; Oya and Okuno, 1975). Antibodies to the NS proteins, including NS1, were also detected in humans who recovered from JE (Kurane, 2002).

JEV-specific T lymphocytes were detected in patients in the early convalescent phase (Konishi et al., 1995). JEV- specific $CD8^+$ human CTLs were elicited by immunization with recombinant attenuated vaccinia virus encoding the preM, E and NS1 genes (Konishi et al., 1998a).

Mouse models have been used to analyze the immune responses to JEV. Infection of mice with JEV induces antibodies to structural and NS proteins; neutralizing antibodies recognize the E protein (Heinz et al., 1983; Rey et al., 1995b). Furthermore, neutralizing epitopes were analyzed using a panel of MAbs against the E protein (Kimura-Kuroda and Yasui, 1986).

Infection by JEV induced virus-specific CTLs (Konishi et al., 1997; Murali-Krishna et al., 1996; Takada et al., 2000). These T cells possessed the $CD3^+ CD4^- CD8^+$ phenotype and an ability to lyse JEV infected cells in a major histocompatibility antigen complex (MHC)-restricted fashion. The proteins recognized by JEV-specific CTLs were different depending on the mouse strains used in the experiments. Virus-specific CTLs raised in BALB/c mice ($H\text{-}2^d$) recognized the E protein, whereas those in C56BL/6 mice ($H\text{-}2^b$) and C3H mice ($H\text{-}2^k$) recognized mainly the NS1 protein (Takada et al., 2000).

### 1.8.4 Protective Immunity Induced by JE Inactivated Vaccine

The main protection mechanism induced by the vaccine is through the production of high levels of neutralizing antibodies, as the inactivated vaccine does not induce high levels of $CD8^+$ CTLs. Although the inactivated vaccine does not contain NS1, antibodies to NS1 were detected in some vaccines. The results suggest that these vaccines were infected with JEV, that virus propagated, and that antibody to NS1 was induced (Kurane, 2002).

It has been demonstrated that immunized mice with undetectable levels of neutralizing antibodies were protected from lethal challenge (Konishi et al., 1999). High levels of neutralizing antibody were produced rapidly in these mice after challenge. It is thus likely that sterile immunity is not established in some of the vaccines, rather they are infected but viruses are eliminated by rapidly produced antibodies before symptoms develop.

### 1.8.5 Neutralization Activities of Murine and Human Sera to JEV Strains

JEV strains are divided into four genotypes according to their nucleotide sequences. The neutralizing activities of antisera raised by one strain of JEV to heterogeneous strains have been studied examining the neutralizing activities of murine and rabbit antisera against homologous and heterologous strains of JEV (Ali and Igarashi, 1997; Ali et al., 1995). Neutralizing titers against heterologous strains were lower than those against homologous strains, and titers were much lower against those strains belonging to different genotypes (Wills et al., 1992).

Similar observations to those in mice were found using human sera from subjects immunized with JE vaccine. It was demonstrated that neutralizing antibody titers induced by the inactivated vaccine were lower to heterologous strains compared with the homologous strain. The neutralizing antibody responses to the Nakayama, JaGar01 and E-50 strains of JEV were compared after immunization with the Nakayama strain vaccine (Juang et al., 1983; Susilowati et al., 1981) and were found to be approximately ten-fold lower against JaGAr-01 and E-50 strains than against the homologous Nakayama strain.

Similar results were demonstrated in Taiwan (Shyu et al., 1997). The neutralizing antibody titers also have been examined after two or three immunizations with Beijing-1 or Nakayama vaccine (Ku et al., 1994). Neutralizing antibody titers were higher against homologous strains than against heterologus strains, although the Beijing-1 vaccine induced higher levels of neutralizing antibody against the Nakayama strain than did the Nakayama vaccine against the Beijing-1 strain. However, the field vaccine efficacy studies in Taiwan (Hsu et al., 1971) and Thailand (Hoke et al., 1988) suggest that protective immunity against heterologous strains is established by immunization with the current inactivated vaccine (Kurane, 2002).

### 1.8.6 Flavivirus Cross-reactive Immunity Induced by JEV

The genus *Flavivirus* includes almost 70 members. These viruses are separated into eight groups based on their serological relatedness (Monath and Heinz, 1996). JEV belongs to the JE antigenic complex with SLEV, MVEV, WNV, KUNV and others.

Neutralizing antibodies induced by JEV neutralized WNV and MVEV to lower but significant levels (Goverdham et al., 1992; Hammon and Sather, 1956). Furthermore, immunized hamsters with JEV were immune not only to JEV, but also to MVEV and WNV (Hammon and Sather, 1956).

Immunization with JEV protected bonnet macaques against WNV, and immunization with WNV reduced the severity of JEV infection (Goverdham et al., 1992). However, It is not known whether immunization with the current JE vaccine protects humans from infection by other viruses included in the JE serocomplex (Kurane, 2002).

In Thailand, immunization with JE vaccine did not significantly reduce the number of patients with DEN fever (Hoke et al., 1988). This may be because antibodies induced by JE vaccine do not neutralize DENV. However, there is a report that prior DEN viral infection moderately reduced the mortality and morbidity of JE patients (Edelman et al., 1975).

## 1.9 JE Vaccination

### 1.9.1 JE Vaccination History

The Russians were the first to practice vaccination against JE in 1941 (Pond and Smadel, 1954). In the United States (US), a formalin inactivated mouse brain vaccine was developed for use in the US Army between 1945 and 1947, and children in Okayama, Japan, between 1946 and 1949 (Pond and Smadel, 1954).

### 1.9.2 JE Vaccines

JE is a vaccine-preventable disease. Three JE vaccines are currently available, namely inactivated mouse brain derived JE vaccine from Nakayama-NIH strain and Beijing-1 strain, inactivated primary BHKC-derived JE vaccine from P3 strain and live attenuated JE vaccine from SA14 strain.

#### Mouse Brain-derived Inactivated JE Vaccine

The only internationally accepted vaccine today is the mouse brain derived inactivated JE vaccine produced using the Nakayama and Beijing-1 strains. Mice are inoculated

intracerebrally with JEV and the brains are harvested when the mice show signs typical of encephalitis (Kitano and Oya, 1996). The brains are triturated in buffered isotonic sodium chloride (Na Cl) solution and centrifuged. The supernatants are treated by alcohol precipitation and with protamine sulphate, and then purified by ultracentrifugation. JEV is inactivated by formalin or any other appropriate reagents of comparable effectiveness. This vaccine, therefore, consists of the E, M, and C proteins (Monath, 2002).

In Japan and the former Soviet Union crude mouse brain vaccines were developed and tested in the late 1930s and in the US during World War II. In Japan purification steps were introduced into the manufacturing process in the 1960s. By 1966, a purified vaccine suitable for children had been developed from infected adult mouse brain tissue and introduced into routine use (Monath, 2002).

Inactivated mouse brain vaccine is prepared either from brain tissue infected with the prototype Nakayama strain originally isolated in 1935 or the Beijing-1 strain originally isolated in 1948 in China. Beijing-1 vaccine has higher potency and elicits a broader neutralizing antibody response across wild-type strains of JEV (Kitano et al., 1986).

The volume for subcutaneous (s.c.) inoculation depends on the vaccine formulation. The vaccine produced from Nakayama strain is given in a volume of 0.5 ml for 1-3 years old children and 1 ml for adults and children older than 3 years. Beijing-1 vaccine is given at one-half these volumes as it is more potent (Monath, 2002).

In JE endemic Asian countries, the primary immunization schedule involves two doses of vaccine administered 1-4 weeks apart, followed by booster doses at 1 year and at variable intervals thereafter (Monath, 2002).

The age for primary immunization varies by country, depending on the force of enzootic transmission and age-specific risk of natural infection. In Japan and South Korea, where JE incidence has declined, vaccination starts at 18 months to 3 years of age, with boosters given at 1 year and subsequently at 3-5 year intervals through age 15 years. In Thailand, vaccination starts at 18 months of age and the second dose is given at 24-36 months of age (Monath, 2002).

The Advisory Committee on Immunization Practices (ACIP) recommends three doses (at 0, 7 and 30 or 0, 7 and 14 days) for primary immunization of travellers, military personnel or persons residing in nonendemic areas (ACIP, 1993).

Mouse brain vaccine is well tolerated, and adverse reactions are of mild degree in more than 90% of subjects. Serious adverse events associated with the vaccine include allergic and neurologic complications.

Allergic effects include hypersensitivity reactions characterized by urticaria, angioedema and bronchospasm are reported (Andersen and Ronne, 1991; Berg et al., 1997; Nazareth et al., 1994; Plesner et al., 2000; Plesner and Ronne, 1997; Ruff et al., 1991). In addition to anaphylactic responses, generalized pruritis has been noted. Most of patients respond well to treatment, but anaphylaxis may be life-threatening, and three deaths have been attributed to the vaccine.

Neurologic effects include encephalitis, encephalopathy, convulsions, peripheral neuropathy, and transverse myelitis were recorded (Ohtaki et al., 1995; Plesner et al., 1996; Tsai et al., 1999).

Use of mouse brain vaccine has been severly restricted due to concern about serious adverse events and a safer vaccine was developed.

Immunogenicity of the mouse brain-derived inactivated vaccine in humans has been studied by many groups. Neutralizing antibodies at 1:10 or greater were induced by three immunizations with this vaccine (Kanamitsu et al., 1970; Minamitani et al., 1974). The levels of antibodies declined to between one-third and one-half of the original levels in 3 years, but the titers were still 1:10 or greater in most vaccines (Akiyama et al., 1996; Takei et al., 1982). Seroconversion rates were 94% after three immunizations with each of Nakayama and Beijing-1 JE vaccines in Thailand (Nimmannitya et al., 1995).

JEV-specific human CTLs were induced in humans by immunization with mouse brain-derived inactivated JE vaccine (Aihara et al., 1998). These CTLs had the $CD3^+$ $CD4^+$ $CD8^-$ phenotype and recognized the E protein. The established $CD4^+$ CTLs clones had two types of

virus specificity: some T-lymphocytes clones were specific for only JEV, and others were cross-reactive for JEV and WNV (Monath, 2002).

Considering the notion that the protective neutralizing antibody level is 1:10, it is expected that the current inactivated JE vaccine will induce protective immunity which lasts for at least 3 years. Protective efficacy has been determined by two studies. The protective efficacy after one or two immunizations was estimated to be 50% and 80%, respectively, in a field study in Taiwan in 1965 (Hsu et al., 1971). In a study in Thailand, Thai children were immunized twice with Nakayama strain vaccine, or with a bivalent vaccine consisting of Nakayama and Beijing-1 strains. The protective efficacy was estimated to be 91% by either protocol (Hoke et al., 1988). Thus, the inactivated JE vaccine was confirmed to be efficacious in protection against JE in the field.

## Inactivated Cell Culture Vaccines

Formalin inactivated unpurified vaccine prepared in primary hamster kidney (PHK) cells infected with P3 strain has been widely used in China since the late 1960s. Two 0.5 ml s.c. doses are administered 1 week apart to children at 6-12 months of age, followed by boosters at 1 year, at school entry and at age 10 years (Monath, 2002).

The immunogenic potency of the inactivated PHK vaccine appears to be modest, with seroconversion rates as low as 60-70% and relatively low antibody levels (Tsai et al., 1999).

In the 1990s, several independent efforts were initiated to develop purified inactivated vaccines in Vero cells using both wild type virus strains and the attenuated SA14-14-2 vaccine strain. The Vero based system has significant advantages in quality, absence of animal proteins and allergens, and more efficient low cost manufacture compared to mouse brain vaccine (Monath, 2002).

In China, a formalin inactivated vaccine has been developed by the National Vaccine and Serum Institute (NVSI), Beijing in Vero cells infected with P3 strain (Ding et al., 1998).

In the US, a formalin-inactivated vaccine has been developed by the Walter Reed Army Institute of Research (WRAIR) in Vero cells infected with the attenuated SA14-14-2 strain and adjuvanted with alum. The main advantage of this approach is that large-scale production of a virulent virus requiring Biosafety level 3 (BL3) containment is avoided.

The vaccine is well tolerated, but immune responses were low with only 40%-70% seroconversion across treatment groups (Monath, 2002). However, after a booster dose, neutralizing antibodies were developed to increase the seroconverison to 80%.

## Live, Attenuated Vaccines

The derivation of the live, attenuated SA14-14-2 vaccine was a significant accomplishment in China (Yu et al., 1988; Yu et al., 1981).

The SA14-14-2 vaccine was licensed in 1988, and it is replacing the older, inactivated PHK vaccine for routine immunization of infants in China with an excellent record of safety and efficacy.

The World Health Organization (WHO) has developed guidelines for live attenuated JE vaccines, with the objective of facilitating the international acceptance of the SA14-14-2 vaccine and access to a low-cost vaccine in developing countries of Southeast Asia.

The biological characteristics of SA14-14-2 have been studied in animals, with particular attention to neurovirulence. The vaccine is attenuated in immunocompetent mice, hamsters, nude and cytoxan treated mice, and in monkeys inoculated by intrathalamic/ intraspinal routes when compared to parental SA14 virus (Tsai et al., 1999). The PrM-E genome sequence and attenuation for mice are stable after serial passages in PHK cells and the attenuated phenotype is also stable after serial passage in suckling mouse brain (Monath, 2002).

The genetic basis for attenuation of SA14-14-2 has been studied. Comparison of the parental SA14 virus and SA14-14-2 vaccine reveals a large number of silent mutations and amino acid changes spread across structural and NS genes (Aihara et al., 1991; Ni et al., 1994; Ni et

al., 1995; Nitayaphan et al., 1990). However, when two other attenuated vaccine viruses developed at earlier stages in the SA14-14-2 lineage (SA14-2-8 and SA14-5-3) are considered, only eight common amino acid differences from SA14 are found. These changes include six in the E glycoprotein (E107, E138, E176, E279, E315 and E439), and single changes in NS2B, NS3 and NS4B. One or more of these mutations are likely to be responsible for the profound phenotypic differences between SA14 and SA14-14-2 (Monath, 2002).

The SA14-14-2 virus is more immunogenic for mice than inactivated PHK vaccine. In addition, studies in mice have demonstrated that immunization with SA14-14-2 cross-protects against JE strains from Thailand, Vietnam and Indonesia, representing diverse JE genotypes (Yu et al., 1989).

SA14-14-2 vaccine showed 98% efficacy across all field trials.

## 1.10 Scope of this Thesis

JE is a complex and fascinating disease; the virus has evolved over time into a flavivirus infection primarily of birds and swine with man as an incidental host. Four genotypes of JEV are presently recognised (representatives of genotype I to IV have been fully sequenced). In addition, a strain of JEV, Muar strain, isolated in Singapore in 1952 from a brain of a patient who originated in Muar, Malaysia may represent a fifth genotype as determined by limited phylogenetic analysis of E gene sequence. I, therefore, decided to determine the complete nucleotide and deduced amino acid sequence of the Muar strain, compare it with other JEV genomes and construct different phylogenetic trees to explore whether the Muar strain is indeed the "missing" fifth genotype of JEV.

It has been reported that the Muar strain has not been isolated from any other area. Why the more ancient lineages of JEV have not spread is uncertain. JEV uses a wide range of mosquito species and vertebrate hosts across Asia, and geographical differences in vector and host availability may explain why the Muar strain has never spread. I examined the virus' complete amino acid sequence to look for molecular determinants that might relate to differences in host preference.

For most of the major human pathogens, we have little idea about where they originated. Because JEV has recently evolved and has so rapidly spread to new areas, tracing its geographical origin has been possible. JE emerged in Japan in 1870 and it has been found across Asia to affect most of China and the Asian subcontinent, all of Southeast Asia, and the Pacific Rim, reaching northern Australian in 1998 and has become the most important cause of epidemic encephalitis worldwide. There is a clear need for a better understanding of the origins and spread of JEV. I, therefore, determined the evolutionary history of JEV isolates and their evolutionary rate per year.

In order to identify the reason for JEV genotype shift and the patterns of genetic change of genotypes, I analyzed the selection pressures on the complete E gene of the JEV five genotypes.

JE remains a major public health problem. Newer treatments for JE which are being considered include treatment with IVIG. There have been case reports of IVIG therapy which have shown it to be beneficial when administered with symptomatic treatment for this disease. In the case report, clinical improvement was rapid and the patient made an almost complete recovery. IVIG has also been assessed recently in a randomised placebo controlled trial for treating encephalitis caused by the flavivirus WNV. I therefore, investigated the role of IVIG from JE-endemic areas in neutralising representative strains of JEV. Given the interest in IVIG as potential treatment for JEV, and the large genetic differences between JEV genotypes, particularly the 5$^{th}$ genotype, I assessed the ability of IVIG from JE-endemic parts of Asia to neutralise representative strains form the 5 genotypes.

## To summarize the aims of the thesis:

1- Determine the complete nucleotide and predicted amino acid sequence of the Muar strain of JEV, compare it with other JEV genomes and determine the nucleotide and amino acid sequence divergence among the Muar strain and other JEV strains.

2- Determine the phylogenetic relationship among the Muar isolate and other JEV strains using both nucleotide and amino acid sequence.

3- Predict the secondary structure of 5` and 3` NCRs of the Muar strain.

4- Determine the evolutionary history of JEV strains.

5- Look at the pattern of the geographical distribution of JEV isolates.

6- Look at the effect of molecular evolution of JEV on the virus spread into new geographical areas and on the host specificity.

7- Determine the selection pressures in E genes of JEV strains belonging to different JEV genotypes to understand the role played by that selection on JEV evolution.

8- Predict the three dimensional structure of the Muar strain E glycoprotein to understand the antigenic and serological properties of this strain.

9- Investigate the impact of the genomic variability seen between JEV genotypes on the neutralization effectiveness of three different IVIGs *in vitro* which are used as a supportive treatment of JEV infection in India.

# CHAPTER 2: MATERIALS AND METHODS

General materials and methods used are described in this chapter. Further specific methods relevant to particular studies are described in subsequent chapters.

## 2.1 Buffers, Reagents and Media

**TAE 1X Buffer**

TAE 50X Buffer (Fisher Scientific) ..... 20 ml

Sterile D. W. ..... 980 ml

**Agarose 1%**

Agrose Multi-Purpose (BIOLINE) ..... 1 g

TAE 1X Buffer ..... 100 ml

**PBS 1%**

PBS Tablets (Fisher Scientific Oxoid Limited) ..... 1 Tablet

Sterile D. W. ..... 100 ml

**Decontaminant for Rnase Away** (Fischer Scientific)

**Nuclease Free Water** (not DEPC treated) (Applied Biosystem)

**Ethanol 190 Proof for Molecular Biology** (Sigma-Aldrich)

**Trypan Blue Solution (0.4%)** (SIGMA)

**Ampicillin Sodium Salt** (SIGMA®) "91.0 – 100.5%"

**Trypsin (10X)** (BioWhittaker)

**Vero Cells Growth Media** (10% FBS)

DMEM (BioWhittaker) ..... 500 ml

FBS (Heat Inactivated) (BioSera) ..... 50 ml

L-Glutamine 200mM (SIGMA®) ..... 5 ml

P/S "10,000 units penicillin and 10 mg streptomycin per ml" (SIGMA®) ..... 5 ml

**Virus Culture Media** (5% FCS)

DMEM (BioWhittaker) ..... 500 ml

FBS (Heat Inactivated) (BioSera) ..... 25 ml

L-Glutamine 200mM (SIGMA®) ..... 5 ml

P/S "10,000 units penicillin and 10 mg streptomycin per ml" (SIGMA®) ..... 5 ml

**Virus Diluent**

MEM 5X (SIGMA life Science) ..... 20 ml

BSA 5% (Fisher Scientific) ..... 20 ml

Na HCO3 7.5% (SIGMA®)..... 4 ml

P/S "10,000 units penicillin and 10 mg streptomycin per ml (SIGMA)" ..... 1 ml

Sterile D. W. ..... 55 ml

**Nutrient Media (2X)**

MEM 5X (SIGMA life Science) ..... 40 ml

FBS (Heat Inactivated) (BioSera) ..... 4 ml

NaHCO3 7.5% (SIGMA®)..... 6 ml

Gentamycin Sulphate (BioWhittaker®) ..... 200 µl

Fungizone 250 µg/ml (Invitrogen) ..... 200 µl

Sterile D. W. ..... 49.6 ml

**Agarose Overlay (2 %)**

Seaplaque Agarose (low melting temperature) (Lonza) ..... 2 g

Sterile D. W. ..... 100 ml

**LB Broth Lennox** (FORMEDIUM ™):

Suspend 20 g in 1 L of distilled de-ionised water

**LB Agar Lennox** (FORMEDIUM ™):

Suspend 35 g in 1 L of distilled de-ionised water

## 2. 2 Primer Design

Primers were designed to amplify overlapping fragments of the genome on the basis of a consensus alignment of complete JEV genomes available through Genbank and on the sequence of the Muar genome already derived. The length of PCR primers ranged from 18 to 22 base pairs (bp). The GC content of the primer ranged between 40-60%. Primer melting temperature ($T_m$) was calculated using this formula; 2 (A+T) + 4 (C+G). Primer annealing temperature (Ta) is usually 5-10 °C below $T_m$.
http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html

Primers designed for the complete genome sequencing of the Muar strain of JEV are outlined in Table 2-1. Detalis of JEV isolates used for primer design and phylogenetic analysis are listed in Table 2-2.

Table 2-1: Designed primers for sequencing of the complete genome of the Muar strain of JEV.

| Oligo Name | Sequence (5′ - 3′) | Product size |
|---|---|---|
| **JEV (4 S)** | AGTTTATCTGTGTGAACTTCTTGG | **1261 bp** |
| **JEV (1265 A)** | AARCCTTGYTTGCACACRTA | |
| **Muar (785 S)** | GGATGCTTGGCAGTAACAAC | **1856 bp** |
| **Muar (2641 A)** | CTCTGTGCACATGCCATAGG | |
| **JEV (1202 S)** | CCACGACTGGAGAAGCYCACAA | **2316 bp** |
| **JEV (3518 A)** | CCACACCTCATCTCTTTTCTTG | |
| **Muar (3125 S)** | CATCACGTCGACTCGAGTGTGG | **1926 bp** |
| **Muar (5051 A)** | CCGATCCTGGATGCAGGTCCAA | |
| **Muar (3829 S)** | GGACTAACCAGGAGAACATTGCTT | **554 bp** |
| **Muar (4383 A)** | CTTCCCAACTAATGTCGGCTGC | |
| **JEV (4421 S)** | GGGAAGCAGCCGGAGGCTAGATGTTAA | **701 bp** |
| **JEV (5122 A)** | TCTTGACGGTCACCTTGCACAATAGCG | |
| **JEV (5005 S)** | GGAACATCCGGCTCACCCAT | **1373 bp** |
| **JEV (6378 A)** | CTTYCTCTCACCCATYCGGG | |
| **Muar (6167 S)** | GGATGGTGAGTATCGATTGAGG | **474 bp** |
| **Muar (6641 A)** | CCACCTGTCATCACGGCTATC | |
| **JEV (6331 S)** | GARGAYAACACYGAGGTRGA | **1322 bp** |
| **JEV (7653 A)** | GTTCTTRATGAGAGTCCAGG | |
| **JEV (6652 S)** | TGACAGGAGGATTCTTCCTGCTCATGATG | **724 bp** |
| **JEV (7376 A)** | AACCATTCCGTCTACGACGGCATTCTT | |
| **Muar (7520 S)** | ATGGTGGCCACTGACGTGCCAG | **3191 bp** |
| **Muar (10,711 A)** | GATCAAGCTGCCACACCAGATGT | |
| **JEV (7601 S)** | TGCGAGGYAGCTACCTRGCT | **1241 bp** |
| **JEV (8842 A)** | GTGACAAGTGGGCCCACAGC | |
| **Muar (8177 S)** | GGCATCAGAGTGGTTGCACCG | **1278 bp** |
| **Muar (9455 A)** | GGTCTTCTCTGGAGATCACATCC | |
| **EMF1** (10,098 S) | TGGATGACGACGGAAGACATG | **730 bp** |
| **VD8** (10,828 A) | GGGTCTCCTCTAACCTCTAG | |
| **Muar (10,220 S)** | GAAGACATCTGGTGTGGCAG | **724 bp** |
| **JEV (10,944 A)** | CCACCAGCTACATGTTTCGGCGCTC | |

*JEV primers were designed by Professor Tom Solomon.
* Muar primers were designed from derived Muar sequence by Manal Mohammed.
* EMF1 and VD8 primers are universal primers for mosquito-borne flaviviruses (Pierre et al., 1994).

Table 2-2. Detalis of isolates used in this study for primer design and phylogenetic analysis. All strains are JEV, except for MVE, SLE and WN. GenBank accession numbers refer to the whole genome or the genes indicated in parenthses. (E, envelope gene). IU, information unavailable.

| Strain | Year | Location | Source | GenBank accession no. |
|---|---|---|---|---|
| 014178 | 2001 | India | Human | EF623987 |
| 04940-4 | 2002 | India | Mosquito | EF623989 |
| 057434 | 2005 | India | Human | EF623988 |
| 2372 | 1979 | Thailand | Human | U70401 (E) |
| 691004 | 1969 | Sri Lanka | Human | Z34097 (E) |
| B58 | 1986 | China | Bat | FJ185036 |
| Beijing-1 | 1949 | China | Human | L48961 |
| Bennett | Before 1951 | Korea | Human | FJ872376 (E) |
| CH1392 | 1990 | Taiwan | Mosquito | AF254452 |
| CH2195LA | 1994 | Taiwan | Mosquito | AF221499 |
| CNS138-11 | 1999 | Malaysia | Human | AY184213 (E) |
| FU | 1995 | Australia | Human | AF217620 |
| GB30 | 1997 | China | Bat | FJ185037 |
| GP05 | 2005 | India | Human | FJ979830 (E) |
| GP78 | 1978 | India | Human | AF075723 |
| HEN0701 | 2007 | China | Swine | FJ495189 |
| HVI | 1958 | Taiwan | Human | AF098735 |
| Ishikawa | 1998 | Japan | Mosquito | AB051292 |
| JaGAr01 | 1959 | Japan | Mosquito | AF069076 |
| JaNAr0102 | 2002 | Japan | Mosquito | AY377577 (E) |
| JaOArS982 | 1982 | Japan | Mosquito | NC_001437 |
| JaOH0566 | 1966 | Japan | Human | AY508813 |
| JEV/sw/Mie/40/2004 | 2004 | Japan | Swine | AB241118 |
| JEV/sw/Mie/41/2002 | 2002 | Japan | Swine | AB241119 |
| JKT1724 | 1979 | Indonesia | Mosquito | U70404 (E) |
| JKT5441 | 1981 | Indonesia | Mosquito | U70406 (E) |
| JKT6468 | 1981 | Indonesia | Mosquito | AY184212 |
| JX61 | 2008 | China | Pig | GU556217 |
| K87P39 | 1987 | Korea | Mosquito | AY585242 |
| K94P05 | 1994 | Korea | Mosquito | AF045551 |
| KPP034-35CT | 1982 | Thailand | Mosquito | U03693 (E) |
| Ling | 1965 | Taiwan | Human | L78128 |
| M15 | 1995 | Australia | Mosquito | L47349 (E) |
| M40 | 1995 | Australia | Mosquito | L47350 (E) |
| M859 | 1967 | Cambodia | Mosquito | U70410 (E) |
| **Muar** | 1952 | Malaysia | Human | **HM596272** |
| MVE 1-51 | 1951 | Australia | Human | AF161266 |
| Nakayama | 1935 | Japan | Human | EF571853 |
| Nakayama- RFVL | IU | Japan | IU | S75726 (E) |
| NJ 2008 | 2008 | IU | IU | GQ918133 |
| NO | 1995 | Australia | Human | L43566 (E) |
| P20778 | 1958 | India | Human | AF080251 |
| P3 | 1950 | China | Mosquito | U47032 |
| PhAn1242 | 1984 | Philippines | Pig | U70417 (E) |
| RP-9 | 1985 | Taiwan | Mosquito | AF014161 |
| SA14 | 1954 | China | Mosquito | U14163 |
| SC04-17 | 2009 | China | Mosquito | GU187972 |
| SH17M07 | 2007 | China | IU | EU429297 |
| SLE MSI.7 | 1975 | United States | Bird | NC_007580 |
| T1P1 | 1997 | Taiwan | Mosquito | AF254453 |
| TC | IU | Taiwan | IU | AF098736 |
| TS00 | 2000 | Australia | Pig | EF434785 (E) |
| VN118 | 1979 | Vietnam | Mosquito | U70420 (E) |
| WN Eg101 | 1950 | Egypt | Human | AF260968 |
| WTP-70-22 | 1970 | Malaysia | Mosquito | U70421 (E) |
| XJ69 | 2007 | China | Mosquito | EU880214 |
| XJP613 | 2007 | China | Mosquito | EU693899 |

# CHAPTER 3: SEQUENCING & PHYLOGENETIC ANALYSIS OF THE COMPLETE GENOME OF THE MUAR STRAIN OF JEV

## 3.1 INTRODUCTION

JEV consists of four genotypes; I, II, III and IV as determined by genetic studies using the nucleotide sequence of C/prM and E genes (Chen et al., 1992; Chen et al., 1990). Representatives of each of these four genotypes have been fully sequenced (Solomon et al., 2003b). In addition, a strain of JEV, Muar strain, isolated in Singapore in 1952 from a brain of a patient who originated in Muar, Malaysia (Hale et al., 1952) may represent a fifth genotype as determined by MAbs reactivities and (Hasegawa et al., 1995; Kobayashi et al., 1984) and limited phylogenetic analysis based on E gene sequence (Hasegawa et al., 1994; Solomon et al., 2003b; Uchil and Satchidanandam, 2001). Figure 3-1 illustrates the geographical distribution of JEV's genotypes.

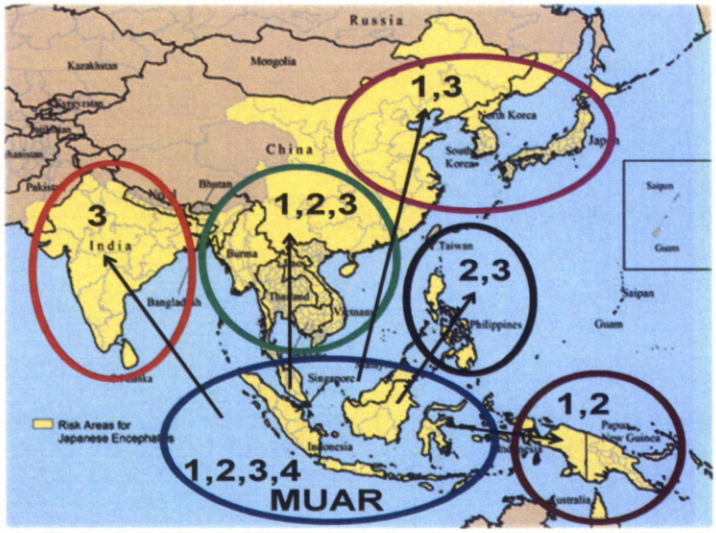


Figure 3-1. The geographical distribution of JEV genotypes and their spread from the Indonesia Malaysia region. Adapted from (Solomon *et al.*, 2003b).

The unavailability of the Muar sequence in computer databases makes the genetic examination of the Muar virus somewhat complicated as we could not determine the relation between Muar and other JEV isolates. It was therefore, decided to determine the complete nucleotide and deduced amino acid sequence of the Muar strain, compare it with other JEV genomes and determine the nucleotide and amino acid sequence divergence among the Muar strain and representative strain of the four JEV genotypes. Also, the phylogenetic relationship among the Muar isolate and other JEV strains was examined using both nucleotide and amino acid sequence.

## 3.2 MATERIALS & METHODS

### 3.2.1 Sequencing of the Muar Isolate Complete Genome

#### *3.2.1.1 Sequencing of the Muar ORF*

The Muar strain of JEV, isolated in Singapore in 1952 from a patient brain who originated in Muar, Malaysia, was obtained from Dr Ichiro Kurane, National Institute of Infectious Diseases, Tokyo, Japan but with unknown passage history.

The Muar virus was inoculated onto confluent monolayers of Vero cells in 5 % FCS. Culture supernatant containing the virus was harvested at 5 to 6 days postinfection, when CPE were observed. Virus was passaged four times and stored at -70 °C.

RNA was extracted by using the QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer's instructions. The extracted RNA was used as a template for the amplification of cDNA by reverse transcriptase-PCR (RT-PCR). Muar RNA was reverse transcribed and was PCR amplified by using Titan One Tube RT-PCR System (Roche) according to manufacturer's instructions. The RT-PCR conditions used are as follows:

| | | | |
|---|---|---|---|
| Reverse transcriptase | 50 °C | 30 min | x1 |
| Denaturing | 94 °C | 2 min | |
| Annealing | 55 °C | 30 sec | |
| Extension | 68 °C | 2 min | |
| Denaturing | 94 °C | 15 sec | x9 |
| Annealing | 55 °C | 15 sec | |
| Extension | 68 °C | 2 min | |
| Denaturing | 94 °C | 15 sec | x25 |
| Annealing | 55 °C | 15 sec | |
| Extension | 68 °C | 2 min Δ 5 sec* | |
| Hold | 4 °C | | |

*Δ 5 sec means cycle elongation of 5 sec for each cycle (e.g., cycle no. 10 has additional 5 sec, cycle no. 11 has additional 10 sec, cycle no. 12 has additional 15 sec).

PCR products were analyzed visually by electrophoresis through ethidium bromide-stained 1% agarose gels under UV light to check the template size and estimate the concentration of the purified cDNA to be sure that it is high enough for the sequencing reaction as determined by the sequencing company Eurofins MWG Operon®; the concentration of template needed to sequence a PCR product depends on the size of the PCR fragment as follows:

| **Template size** | **Concentration** |
|---|---|
| 200-500 bp | 5-16 ng/µl |
| 500-1000 bp | 16-42 ng/µl |
| 1000-2000 bp | 42-83 ng/µl |
| > 2000 bp | 83-100 ng/µl |

The template size and concentration of the RT PCR products was determined by using 1 Kb DNA ladder (BioLabs) visualized by ethidium bromide staining on a 1% TAE agarose gel. RT-PCR products were purified by using the QIAquick Gel Extraction Kit (QIAGEN). Purified cDNA was directly sequenced in both directions by using the appropriate sense and antisense primers. Amplicons were sequenced on both strands via an automated ABI 3730XL sequencer by Eurofins MWG Operon® Company.

### *3.2.1.2 Sequencing of The Muar 5′ NCR*

Sequencing of 5′ NCR was determined by using 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) and amplification of target cDNA was done by using Expand High Fidelity plus PCR System, dNTPack (Roche).

PCR products were analyzed visually by electrophoresis through ethidium bromide-stained 1% agarose gels under UV light to estimate their concentrations for sequencing by Eurofins MWG Operon® Company. RT-PCR products were purified by using the QIAquick Gel Extraction Kit (QIAGEN).

Primers used for 5′ NCR sequencing were:

1-

2- Primer for the PCR of cDNA:

Muar (1265 A): {5′ GTGGCGTGCGCCTCTTCAAA 3′}

2- Primer for the nested amplification step:

Muar (194 A): {5′ TACCCTCTTCACTCCCACTAGTG 3′}

### *3.2.2.3 Sequencing of The Muar 3′ NCR*

3′ NCR sequence was obtained by using *E. coli* poly (A) polymerase (BioLabs) to add poly (A) tail to the Muar mRNA. Subsequently, the poly (A) mRNA was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) and was PCR amplified by HotStarTaq Master Mix Kit (Qiagen) using a gene-specific primer.

Primers used for 3′ NCR sequencing were:

1- Primer to add poly (A) tail to the mRNA:

3′ RACE AUAP: {5′ GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT 3′}

2- Primer for the first PCR:

EMF1 (10,098 S): {5′ TGGATGACGACGGAAGACATG 3′}

3- Primer for the nested amplification step:

Muar (10,220 S): {5′ GAAGACATCTGGTGTGGCAG 3′}

RT-PCR products were purified and their concentrations were estimated before sequencing as described for 5′ NCR.

3′ UTR was sequenced as following:

| | | |
|---|---|---|
| In PCR tube, add: | Purified RNA | 21 µl |
| | 3' RACE AUAP (20 µM) | 5 µl |

Then run RT cycle (65 $^{0}$C for 10 minutes) and put on ice for 5 minutes.

| | | |
|---|---|---|
| Then add: | Transcriptor 5X Buffer | 8 µl |
| | Rnase inhibitor | 1 µl |
| | dNTP* | 4 µl |
| | Transcreptor RT | 1 µl |

Then run RT cycle (55$^{0}$ C for 1 hour) followed by PCR purification.

*dNTP Mix, PCR Grade (200 µl) "QIAGEN": Mix containing 10 mM each of dATP, dCTP, dGTP, and dTTP (1 x 200 µl)

**Two PCR were done on the purified cDNA;**

**1st PCR**:

| | |
|---|---|
| Hotstart PCR buffer (2X) | 25 µl |
| 3' RACE AUAP (20 µM) | 1 µl |
| EMF1 (10,098 S) (10 µM) | 2 µl |
| cDNA | 10 µl |
| Rnase free water | 12 µl |

Then run hot PCR cycle for 35 cycles.

**2nd PCR**:

Same as the 1st PCR but another specific primer was used; Muar (10,220 S) instead of EMF1 (10,098).

RT-PCR products were analyzed visually by electrophoresis through ethidium bromide-stained 1% agarose gels under UV light to estimate their concentrations for sequencing by Eurofins MWG Operon® Company. RT-PCR products were purified by using the QIAquick Gel Extraction Kit (QIAGEN).

### 3.2.2 Cloning of PCR products

If there were differences between sequence results, cloning of that region was done using TA Cloning® Kit (Invitrogen) following the manufacturer's instructions.

Ten colonies for each reaction were picked and screened by PCR to confirm they contained an insert of the appropriate size using the Qiagen HotStarTaq Master Mix Kit (Qiagen) according to the manufacturer's instructions.

Specific primers for these regions of the PCR products were used with the following PCR conditions: An initial activation of 95 °C for 15 minutes followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing 68 °C for 1 minute, extension at 72 °C for 1 minute and a final extension at 72 °C for 10 minutes.

Positive colonies containing inserts of the predicted size were then incubated overnight in LB medium containing 100 μg/ml Ampicillin for plasmid extraction. Incubation was done at 37 °C with vigorous shaking at 250 rpm.

Plasmid was extracted using QIAprep® Miniprep Kit (QIAGEN) as described in the protocol.

Plasmids DNA were sequenced on both strands using an automated ABI 3730XL sequencer by the Eurofins MWG Operon® Company.

### 3.2.3 Muar Genomic Sequence Analysis

The Muar strain was sequenced, as described above. The complete genome of Muar strain was compiled by alignment with other JEV complete genome strains available at GenBank (Table 2-2), using the Vector NTI suite software package (version 10; Informax Inc.).

ClustalX (1.81) (Thompson et al., 2002) was used for the alignment of the Muar nucleotide and derived amino acid sequences with other JEV strains.

Percentage differences between the complete genomic sequence and the derived polyprotein of the Muar strain and representative strain of JEV genotypes I, II, III and IV were calculated by using P-distance in MEGA software (version 4.0) (Tamura et al., 2007).

The primary structure of the conserved 5′ and 3′ NCRs of JEV five genotypes and other three flaviviruses belonging to the JE serocomplex; MVEV, WNV and SLEV was compared. Also, the possible folding of these NCRs was predicted using mfold web server using the default folding conditions (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form).

### 3.2.4 Selection of the Best-fit Model for JEV Genome Nucleotide Substitution

Selection of the best-fit model for JEV genome nucleotide substitution was carried out by ModelTest 3.7 (version 0.1.1).

### 3.2.5 Phylogenetic Analysis of the Muar Strain of JEV

Phylogenetic analysis was performed using MP, ML and NJ methods on PAUP* (version 4.04b 10) (Wilgenbusch and Swofford, 2003) using nucleotide and derived polyprotein sequences of C, PrM, E, NS3, NS5 and ORF as well as the nucleotide sequences of the 5' UTR and 3' NCR and the complete genome.

ClustalX was used to create a multiple alignment from the sequence in FASTA format. Pairwise alignment parameters were identified as slow-accurate, with gap opening penalty (15.00) and gap extension (6.66). In multiple alignment parameters, gap opening and gap extension penalties were the same as for the pairwise alignment. Output format option was identified as nexus format which is required for the phylogenetic analysis by PAUP.

In PAUP software, the first step is to define optimality criteria either parsimony, likelihood or distance then heuristic search was selected to perform the search strategy. Bets trees will be displayed and described.

The best-fit evolutionary model used in the ML phylogentic analysis was general time reversible (GTR) + Gamma + invariant model.

The robustness of phylograms was evaluated by 1,000 bootstrap replicates. All trees were visualised using FigTree (version 1.2.2).

## 3.3 RESULTS

### 3.3.1 Determination of the Nucleotide Sequence of the Complete Genome of The Muar Strain of JEV

The complete nucleotide sequence of the Muar strain of the JEV genome RNA was determind using RT PCR. All RT PCR products were analyzed visually by electrophoresis through ethidium bromide-stained 1% agarose gels under UV light and compared with a 1 Kb DNA ladder to determine their sizes. Figures 3-2 to 3-8 show agarose gel of different PCR products of the Muar strain of JEV compared to a 1 Kb DNA ladder (M).

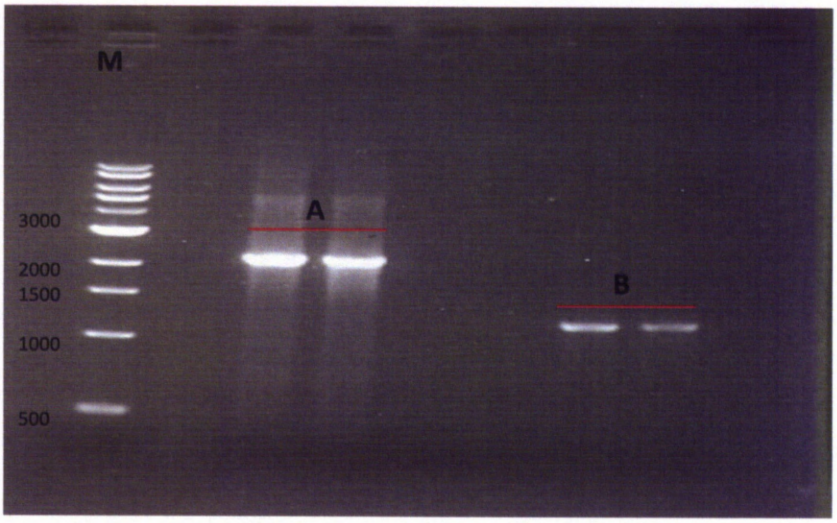


Figure 3-2. Agarose gel electrophoresis of the RT PCR products of the Muar isolate of JEV compared to 1 Kb DNA ladder (M). 2316 bp produced from using primers 1202s and 3518a (A), which amplified part of E and NS1 genes, and 1261 bp produced from using primers 4s and 1265a (B), which amplified part of 5' NCR, C and prM genes and part of the E gene.

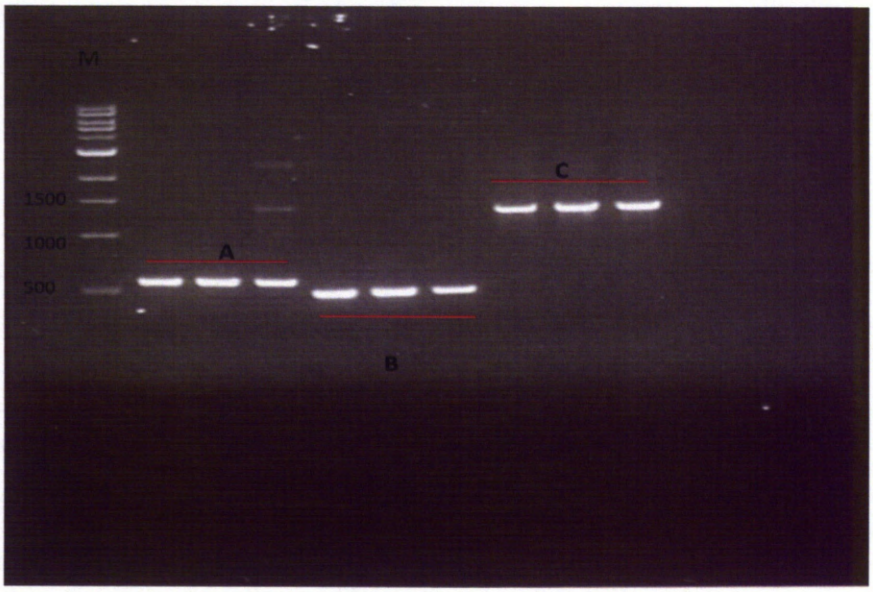


Figure 3-3. Agarose gel electrophrosis of the RT PCR products of the Muar isolate of JEV compared to 1 Kb DNA ladder (M). 554 bp produced from using primers 3829s and 4383a (A), which amplified part of NS2 gene, 474 bp produced from using primers 6167s and 6641a (B), which amplified part of NS3 and NS4 genes, and 1856 bp produced from using primers 785s and 2641a (C), which amplified the E gene.

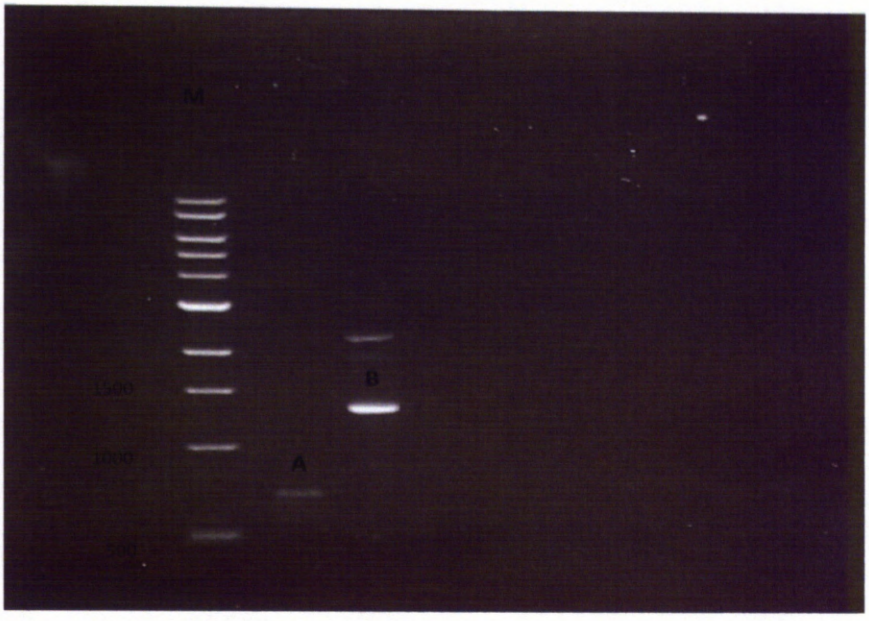


Figure 3-4. Agarose gel electrophoresis of the RT PCR products of the Muar isolate of JEV compared to 1 Kb DNA ladder (M). 724 bp produced from using primers 6652s and 7376a (A), which amplified part of NS4 gene and 1373 bp produced from using primers 5005s, and 6378a (B), which amplified part of NS3 and NS4 genes.

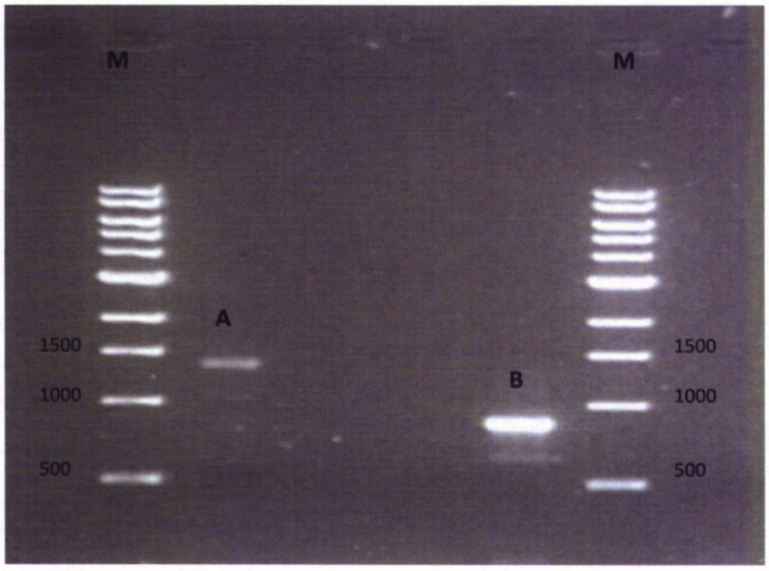


Figure 3-5. Agarose gel electrophoresis of the RT PCR products of the Muar isolate of JEV compared to 1 Kb DNA ladder (M). 1322 bp produced from using primers 6331s and 7653a (A), which amplified part of NS3 and NS4 genes and 730 bp produced from using EMF, and VD8 primers (B), which amplified part of NS5 gene and part of 3' NCR.

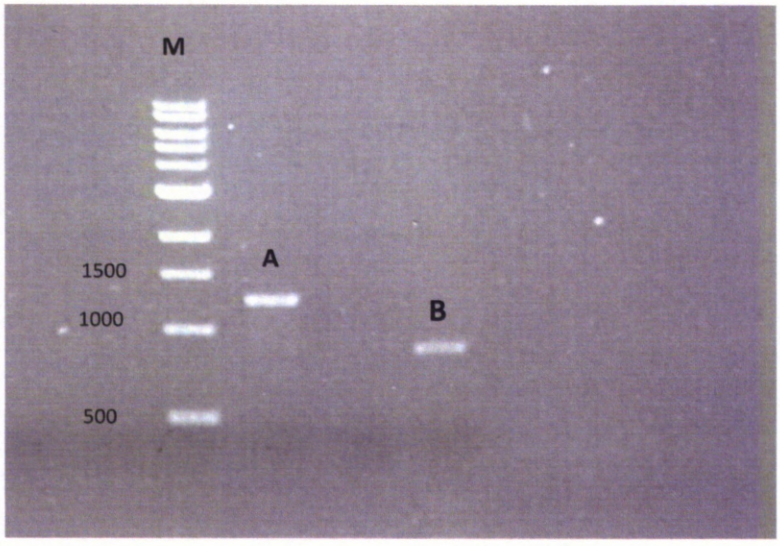


Figure 3-6. Agarose gel electrophoresis of the RT PCR products of the Muar isolate of JEV compared to 1 Kb DNA ladder (M). 1241bp produced from using primers 7601s and 8842a (A), which amplified part of NS4 and NS5 genes, and 724 bp produced from using primers 10,220s and 10,944a (B), which amplified NS5 gene and part of 3' NCR.

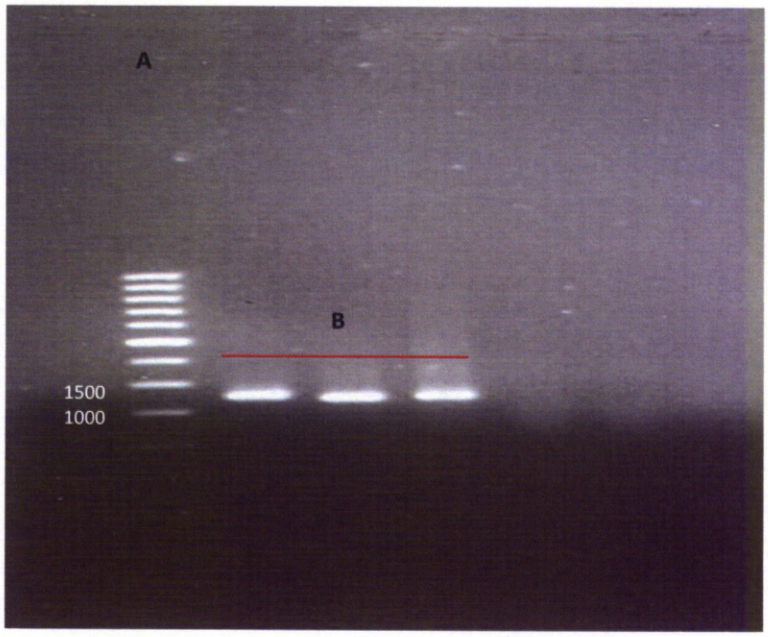


Figure 3-7. Agarose gel electrophoresis of the RT PCR products of the Muar isolate of JEV compared to 1 Kb DNA ladder (M). 1265 bp produced from using primers AUAP and 1265a (A), which amplified the 5' NCR.

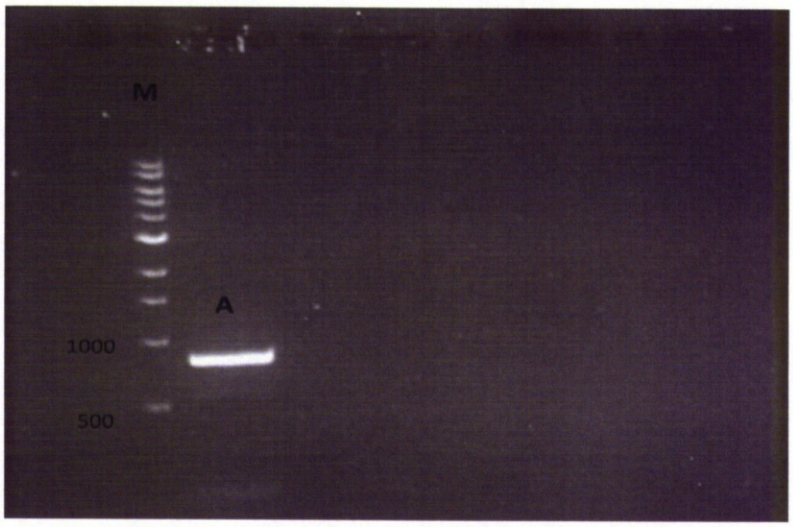


Figure 3-8. Agarose gel electrophoresis of the RT PCR products of the Muar isolate of JEV compared to 1 Kb DNA ladder (M). 980 bp produced from using primers EMF and 3' RACE (A), which amplified the 3' NCR.

The Muar virus genome was 10,988 nucleotides long. The JEV isolate Muar was assigned GenBank database accession no. **HM596272**.

The genome organization of the Muar strain resembles that of other known JE viruses. The RNA genome contains a single ORF extending from nucleotide 96 to nucleotide 10,394, encoding 3,432 amino acids similar in length to those reported for other JE viruses. This ORF is flanked by the 5' and 3' NTRs of 95 and 593 nucleotides, respectively. The length of the 5' NTR is identical with that reported for other JE viruses and the 5' cap is followed by the conserved dinucleotide sequence AG. However, alignment of the 3' NTR sequences of the Muar strain with other JEV strains revealed the presence of an extra 7 to 25 nucleotides in the 3' NTR of the Muar strain genome, located at the 5' side of the 3' NTR.

Like other genomic RNAs of mosquito-borne flaviviruses including JEV, the Muar strain appears to lack a 3'-terminal poly (A) tract and instead to terminate with the conserved dinucleotide CU as shown at the end of the nucleotide sequence of 3' NCR in appendix A.

Processing of the JEV polyprotein generates 10 membrane associated viral proteins (three structural and seven NS proteins), and two polypeptide cleavage fragments. The order of proteins encoded in the long ORF is 5'- C- prM (M)- E- NS1- NS2A- NS2B- NS3- NS4A- NS4B- NS5- 3'. Annotation of the Muar complete genome was determined for other JEV strains (Sumiyoshi et al., 1987) as following:

| | |
|---|---|
| **5' NCR** | 1-95 |
| **C** | 96-476 |
| **prM** | 477-977 |
| **E** | 978-2477 |
| **NS1** | 2478-3533 |
| **NS2** | 3534-4607 |
| **NS3** | 4608-6464 |
| **NS4** | 6465-7679 |
| **NS5** | 7680-10394 |
| **3' NCR** | 10395-10988 |

The complete nucleotide sequence of the Muar strain is given as appendix A.

### 3.3.2 Determination of the Amino Acid Sequence of the ORF of The Muar Strain of JEV

The nucleotide sequence of the Muar strain was translated to the amino acids using ExPASy Proteomics Server (http://expasy.org/tools/dna.html).

The Muar strain has a single ORF with conserved cleavage sites with other JEV strains available on GenBank. Like other JEV strains, Muar has three structural proteins; C, prM and E and seven NS proteins; NS1, NS2A and NS2B, NS3, NS4A and NS4B and NS5.

The deduced amino acid sequence of the Muar strain is given as appendix B.

### 3.3.3 Determination of the Nucleotide and Amino Acid Sequence Divergence Percentage between the Muar Strain and Representative Strains of the Four JEV Genotypes

Comparison of the Muar nucleotide and amino acid sequences with a fully sequenced representative of GI (K94P05) "GenBank accession number: AF045551", GII (FU) "GenBank accession number: AF217620", GIII (Nakayama) "GenBank accession number: EF571853"and GIV (JKT6468) "GenBank accession number: AY184212" showed Muar to have the least similarity, with nucleotide divergence ranging from 20.2% to 21.2%, and protein divergence from 8.5% to 9.9% as shown in Table 3-1.

Analysis by individual genes showed that Muar has 4.2 - 9.5 % nucleotide difference in the 5' NCR from other JEV strains (Table 3-2), 19.2 - 21.5% in the C (Table 3-3), 19.8 - 23.6% in the prM gene (Table 3-4), 22.1 – 23.8% in the E gene (Table 3-5), 21.1 - 22.5% in the NS1 gene (Table 3-6), 21.1 – 22.9% in the NS2 gene (Table 3-7), 19.6 – 20.8% in the NS3 gene (Table 3-8), 7.1 – 8.3% in the NS4 gene (Table 3-9), 19.5 - 20.9 %) in the NS5 gene (Table 3-10) and 13.6 –15.4% nucleotide difference in the 3' NCR (Table 3-11).

**Table 3-1. Nucleotide and amino acid sequence divergence for the complete genome of the Muar strain and representative strains of the four JEV genotypes.**

| | | Divergence (%) with: | | | | |
|---|---|---|---|---|---|---|
| Strain | Genotype | K94P05 | FU | Nakayama | JKT6468 | Muar |
| K9P05 | I | | 10.3 | 10.8 | 16.8 | 21.2 |
| FU | II | **2.1** | | 10.5 | 16.1 | 21.1 |
| Nakayama | III | **3.1** | **2.2** | | 15.1 | 20.2 |
| JKT6468 | IV | **6.2** | **5.3** | **4.8** | | 21 |
| Muar | V | **9.9** | **8.6** | **8.5** | **9.2** | |

Nucleotide divergence is in lightface type; amino acid divergence is in bold face type.

**Table 3-2. Nucleotide sequence divergence for 5' NCR of the Muar strain and representative strains of the four JEV genotypes.**

| | | Divergence (%) with: | | | | |
|---|---|---|---|---|---|---|
| Strain | Genotype | K94P05 | FU | Nakayama | JKT6468 | Muar |
| K94P05 | I | | 2.1 | 4.2 | 8.4 | 4.2 |
| FU | II | | | 4.2 | 6.3 | 4.2 |
| Nakayama | III | | | | 8.4 | 5.3 |
| JKT6468 | IV | | | | | 9.5 |
| Muar | V | | | | | |

**Table 3-3. Nucleotide and amino acid sequence divergence for the C gene of the Muar strain and representative strains of the four JEV genotypes.**

| | | Divergence (%) with: | | | | |
|---|---|---|---|---|---|---|
| Strain | Genotype | K94P05 | FU | Nakayama | JKT6468 | Muar |
| K94P05 | I | | 9.7 | 7.6 | 16.8 | 19.9 |
| FU | II | **4.7** | | 7.6 | 15.7 | 19.7 |
| Nakayama | III | **5.5** | **4.7** | | 15 | 19.2 |
| JKT6468 | IV | **18.9** | **18.9** | **18.1** | | 21.5 |
| Muar | V | **19.7** | **18.9** | **18.9** | **26** | |

Nucleotide divergence is in lightface type; amino acid divergence is in bold face type.

**Table 3-4. Nucleotide and amino acid sequence divergence for the prM gene of the Muar strain and representative strains of the four JEV genotypes.**

| | | Divergence (%) with: | | | | |
|---|---|---|---|---|---|---|
| Strain | Genotype | K94P05 | FU | Nakayama | JKT6468 | Muar |
| K94P05 | I | | 12.2 | 12.6 | 18.4 | 19.8 |
| FU | II | **3** | | 13.8 | 20.4 | 23.2 |
| Nakayama | III | **3.6** | **2.4** | | 17.8 | 22 |
| JKT6468 | IV | **9.6** | **9.6** | **8.4** | | 23.6 |
| Muar | V | **10.2** | **10.8** | **11.4** | **16.8** | |

Nucleotide divergence is in lightface type; amino acid divergence is in bold face type.

**Table 3-5. Nucleotide and amino acid sequence divergence for the E gene of the Muar strain and representative strains of the four JEV genotypes.**

| | | Divergence (%) with: | | | | |
|---|---|---|---|---|---|---|
| Strain | Genotype | K94P05 | FU | Nakayama | JKT6468 | Muar |
| K94P05 | I | | 11 | 12.2 | 18.4 | 23.8 |
| FU | II | **2** | | 11.6 | 18.1 | 22.9 |
| Nakayama | III | **2.6** | **2.6** | | 16.8 | 22.1 |
| JKT6468 | IV | **5.2** | **5.6** | **5.6** | | 22.8 |
| Muar | V | **9** | **8.8** | **8.8** | **9.4** | |

Nucleotide divergence is in lightface type; amino acid divergence is in bold face type.

**Table 3-6. Nucleotide and amino acid sequence divergence for the NS1 gene of the Muar strain and representative strains of the four JEV genotypes.**

| | | Divergence (%) with: | | | | |
|---|---|---|---|---|---|---|
| Strain | Genotype | K94P05 | FU | Nakayama | JKT6468 | Muar |
| K94P05 | I | | 11.4 | 11.8 | 17.8 | 22.5 |
| FU | II | **5.4** | | 9.9 | 16.7 | 22.4 |
| Nakayama | III | **4.3** | **3.7** | | 14.4 | 21.1 |
| JKT6468 | IV | **7.4** | **7.7** | **5.1** | | 21.8 |
| Muar | V | **10.5** | **9.4** | **8.2** | **8.2** | |

Nucleotide divergence is in lightface type; amino acid divergence is in bold face type.

**Table 3-7. Nucleotide and amino acid sequence divergence for the NS2 gene of the Muar strain and representative strains of the four JEV genotypes.**

| | | Divergence (%) with: | | | | |
|---|---|---|---|---|---|---|
| Strain | Genotype | K94P05 | FU | Nakayama | JKT6468 | Muar |
| K94P05 | I | | 9.8 | 11.5 | 18.3 | 22.9 |
| FU | II | **2.8** | | 10.6 | 15.5 | 22 |
| Nakayama | III | **2.5** | **3.1** | | 15.8 | 21.6 |
| JKT6468 | IV | **5.6** | **5** | **4.7** | | 22.1 |
| Muar | V | **13.1** | **11.5** | **11.7** | **11.7** | |

Nucleotide divergence is in lightface type; amino acid divergence is in bold face type.

**Table 3-8. Nucleotide and amino acid sequence divergence for the NS3 gene of the Muar strain and representative strains of the four JEV genotypes.**

| | | Divergence (%) with: | | | | |
|---|---|---|---|---|---|---|
| Strain | Genotype | K94P05 | FU | Nakayama | JKT6468 | Muar |
| K94P05 | I | | 11.1 | 12.7 | 15.6 | 20.7 |
| FU | II | **2.7** | | 11.9 | 16.4 | 20.8 |
| Nakayama | III | **3.2** | **1.1** | | 15 | 19.6 |
| JKT6468 | IV | **4.5** | **2.6** | **2.6** | | 20.2 |
| Muar | V | **7.1** | **5.2** | **5.2** | **5.7** | |

Nucleotide divergence is in lightface type; amino acid divergence is in bold face type.

**Table 3-9. Nucleotide and amino acid sequence divergence for the NS4 gene of the Muar strain and representative strains of the four JEV genotypes.**

| | | Divergence (%) with: | | | | |
|---|---|---|---|---|---|---|
| Strain | Genotype | K94P05 | FU | Nakayama | JKT6468 | Muar |
| K94P05 | I | | 1.3 | 1.8 | 4.3 | 8.1 |
| FU | II | **1.2** | | 1.5 | 4.5 | 7.6 |
| Nakayama | III | **1.7** | **1.5** | | 4.3 | 7.1 |
| JKT6468 | IV | **4.2** | **4.5** | **4.2** | | 8.3 |
| Muar | V | **7.9** | **7.4** | **6.9** | **8.2** | |

Nucleotide divergence is in lightface type; amino acid divergence is in bold face type.

**Table 3-10. Nucleotide and amino acid sequence divergence for the NS5 gene of the Muar strain and representative strains of the four JEV genotypes.**

| | | Divergence (%) with: | | | | |
|---|---|---|---|---|---|---|
| Strain | Genotype | K94P05 | FU | Nakayama | JKT6468 | Muar |
| K94P05 | I | | 10.1 | 9.6 | 16.7 | 20.9 |
| FU | II | **4.1** | | 9.9 | 15.2 | 20.9 |
| Nakayama | III | **3.3** | **1.7** | | 14.3 | 19.5 |
| JKT6468 | IV | **6.1** | **4** | **3.5** | | 19.7 |
| Muar | V | **10** | **8** | **8** | **7.5** | |

Nucleotide divergence is in lightface type; amino acid divergence is in bold face type.

**Table 3-11. Nucleotide sequence for the 3' NCR of the Muar strain and representative strains of the four JEV genotypes.**

| | | Divergence (%) with: | | | | |
|---|---|---|---|---|---|---|
| Strain | Genotype | K94P05 | FU | Nakayama | JKT6468 | Muar |
| K94P05 | I | | 5.7 | 5.7 | 12.7 | 15.4 |
| FU | II | | | 4.4 | 10.8 | 13.6 |
| Nakayama | III | | | | 8.8 | 14.7 |
| JKT6468 | IV | | | | | 14.5 |
| Muar | V | | | | | |

Nucleotide divergence is in lightface type; amino acid divergence is in bold face type.

### 3.3.4 Primary and Secondary structures of 5' and 3' NCR of the Muar strain

Alignment of the terminal nucleotide sequence of the 5' and 3' NCRs of genotype V, the Muar strain, with the other JEV genotypes showed very high homology as shown in Figure 3-9.

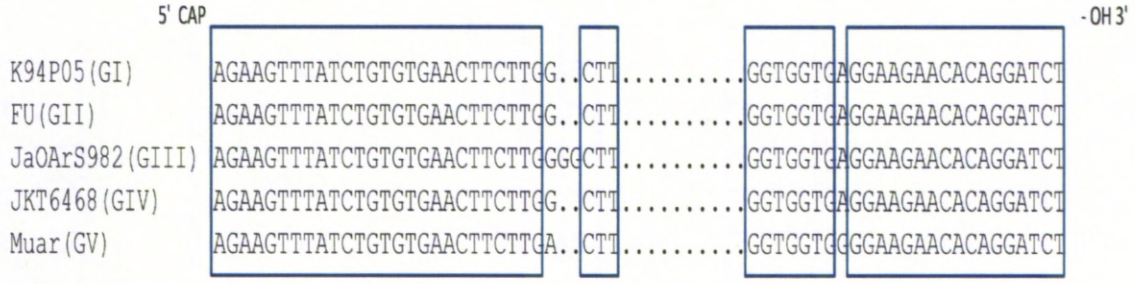


Figure 3-9. Nucleotide sequence homology of 5' and 3' terminal sequences among JEV genotypes. Identical residues among the viruses are boxed.

*Middle residues between the 5' and 3' terminal sequences are not shown here and they are represented by dots.

Furthermore, alignment of the terminal nucleotide sequence of the 5' and 3' NCRs of JE serocomplex; JEV, MVEV, WNV and SLEV showed high homology as shown in Figure 3-10.

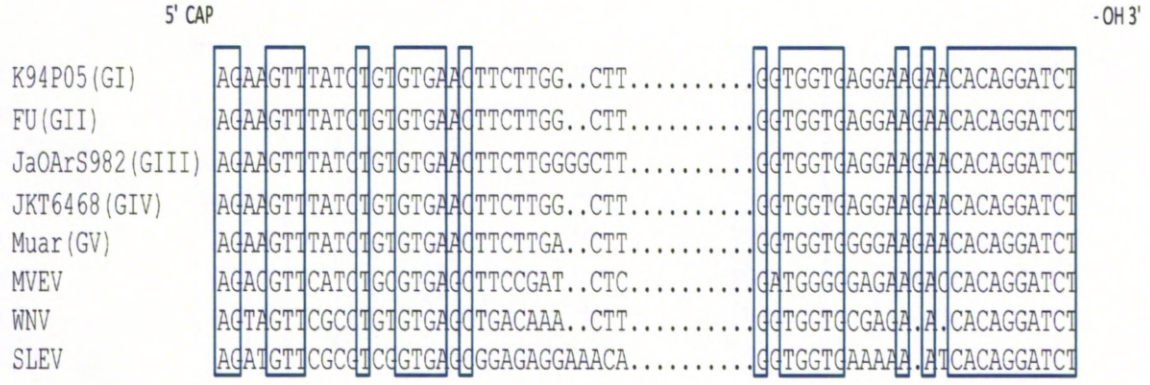


Figure 3-10. Nucleotide sequence homology of 5' and 3' terminal sequences among JE sercomplex; JEV (five genotypes), MVEV, WNV and SLEV. Identical residues among the viruses are boxed.

*Middle residues between the 5' and 3' terminal sequences are not shown here and they are represented by dots.

Similarities in the secondary structures of the 3' NCR of JEV genotypes III and V were also noted and are shown in Figure 3-11.

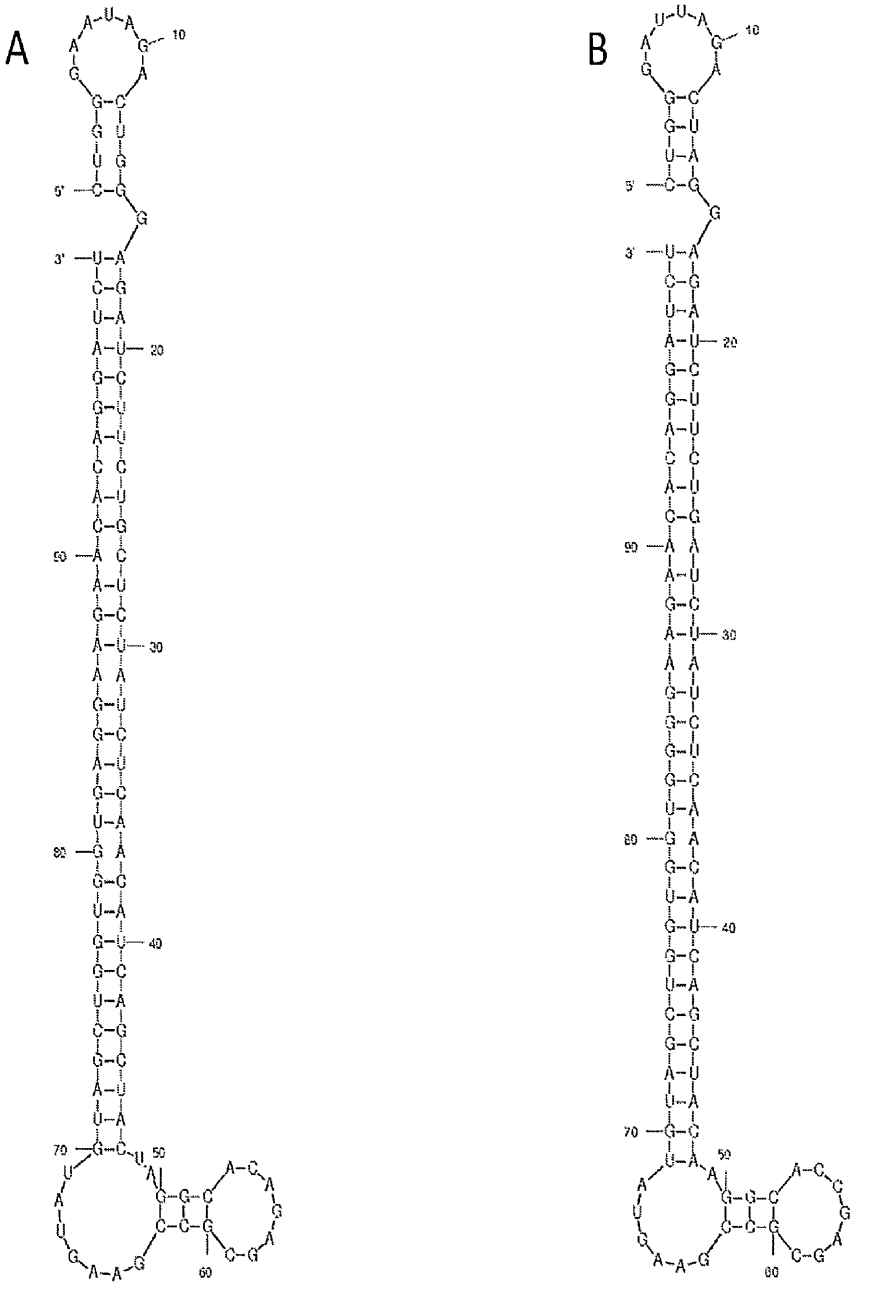


Figure 3-11. Proposed secondary structure for sequences of the part of 3' NCR of JEV. The sequence used for construction of the secondary structure model is from the genotype III, JaOArS982 strain (A) and genotype V, Muar strain (B).

*dG means the minimal folding free energy; the lower dG means the higher possibility of this structure.

Another secondary structure shown in Figure 3-12 is also postulated (Sumiyoshi et al., 1987). The structure base-pairing between 5' and 3' terminal sequences, is also noted in genotypes III and V of JEV.

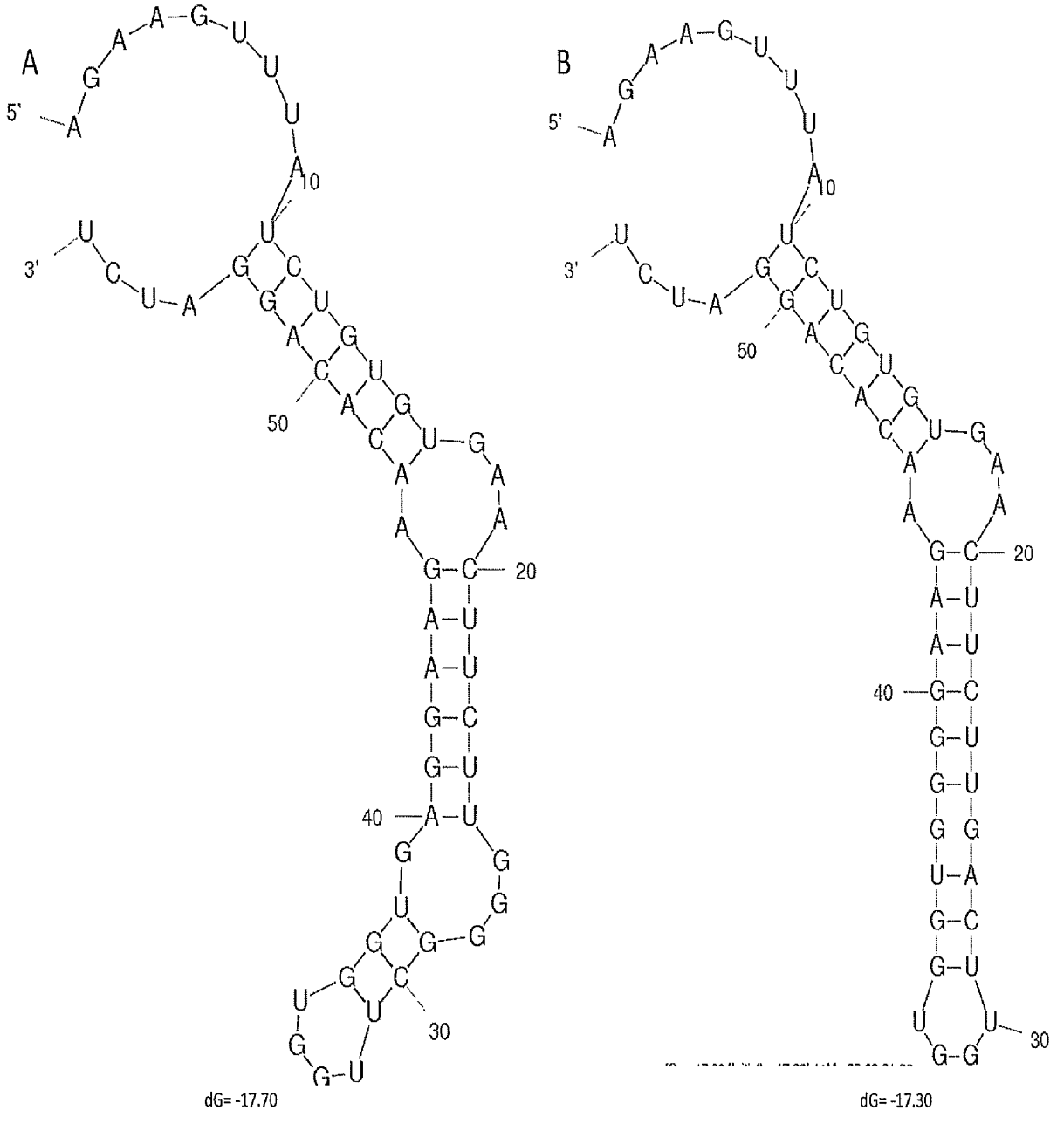


Figure 3-12. Proposed secondary structure for sequences of 5' and 3' NCRs terminal sequences of JEV. The sequence used for construction of the secondary structure model is from the genotype III, JaOArS982 strain (A) and genotype V, Muar strain (B).

*dG means the minimal folding free energy; the lower dG means the higher possibility of this structure.

### 3.3.5 Phylogenetic Analysis among the Muar Strain of JEV and Other JEV Four Genotypes Strains

NJ, MP and ML methods based on both nucleotide and amino acid sequences were used to determine the relation between the Muar strain and other JEV isolates. Phylogenetic analyses were similar and clearly revealed that the Muar isolate represents the fifth genotype of JEV with 100% bootstrap support. Therefore, to avoid presenting similar trees, only NJ trees of the nucleotide sequence of the complete genome and different genes as well as ML of the nucleotide sequence of the E and NS5/ 3′ NCR genes will be presented in this chapter.

NJ phylogeney based on the nucleotide sequence of 5' and 3' NCRs as shown in Figure 3-13 and Figure 3-14, C gene as shown in Figure 3-15, prM gene as shown in Figure 3-16, E gene as shown in Figure 3-17, NS1 gene as shown in Figure 3-18, NS2 gene as shown in Figure 3-19, NS3 gene as shown in Figure 3-20, NS4 gene as shown in Figure 3-21, NS5 gene as shown in Figure 3-22 and the complete genome as shown in Figure 3-23 show that Muar isolate represents a fifth genotype of JEV as it is always diverging at the basal node of all NJ trees, apart from Figure 3-13, before the other four known genotypes with 100% bootstrap support.

ML phylogeney also confirms that the Muar strain represents the fifth genotype of JEV as shown in Figure 3-24 based on the nucleotide sequence of (A) E genes and (B) NS5/ 3′ NCR genes with 100% and 98% bootstrap support respectively.

Thus Muar isolate was placed into a new fifth JEV genotyope based on NJ, ML and MP phylogenetic analyses in this study and previous phylogenetic analysis by Uchil and Satchidanandam (Uchil and Satchidanandam, 2001) and Solomon et al. (Solomon *et al.*, 2003b).

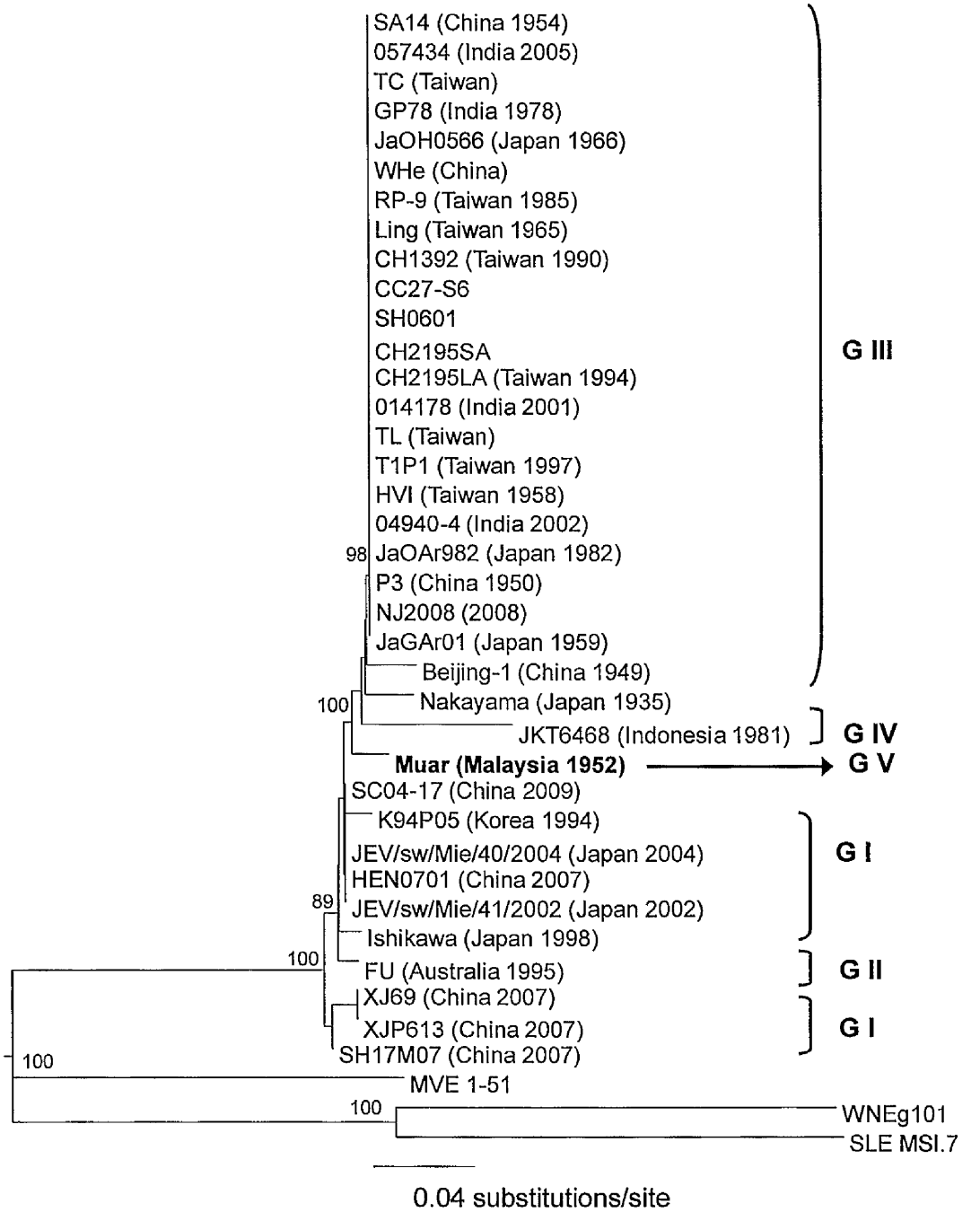


Figure 3-13. A neighbour-joining phylogenetic tree of the JEV **5´NCR** genes, rooted by using a representative strain from other viruses in the JE serogroup (MVEV, SLE and WNV). The Muar isolate is shown with an arrow as a genotype V. Genotypes are given on the right of each tree. Bootstrap support values, given as a percentage of 1,000 replicates, are shown. Country and year of isolation for each strain used are shown in brackets.

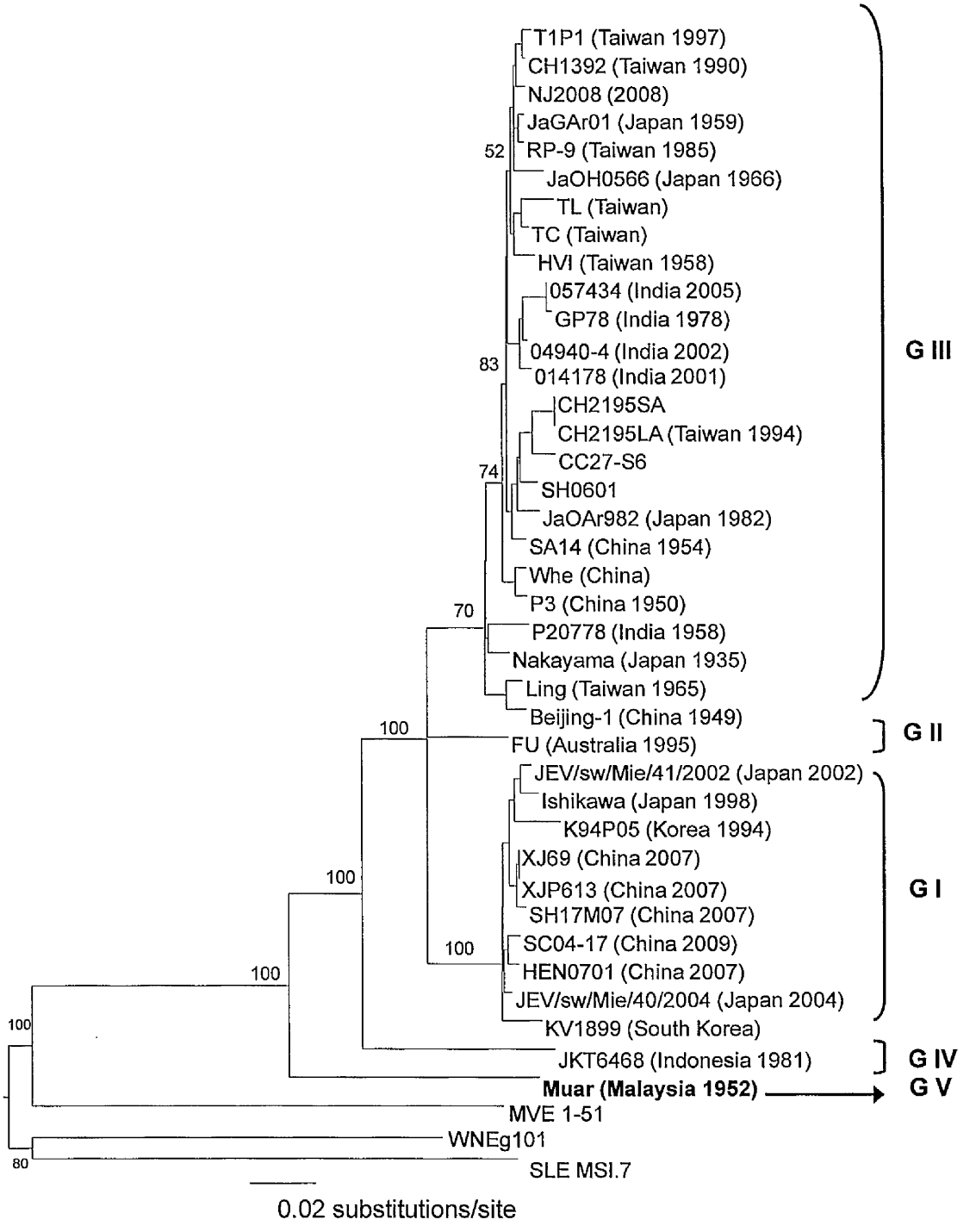


Figure 3-14. A neighbour-joining phylogenetic tree of the JEV **3′NCR** genes, rooted by using a representative strain from other viruses in the JEV serogroup (MVEV, SLE and WNV). The Muar isolate is shown with an arrow as a genotype V. Genotypes are given on the right of each tree. Bootstrap support values, given as a percentage of 1,000 replicates, are shown. Country and year of isolation for each strain used are shown in brackets.

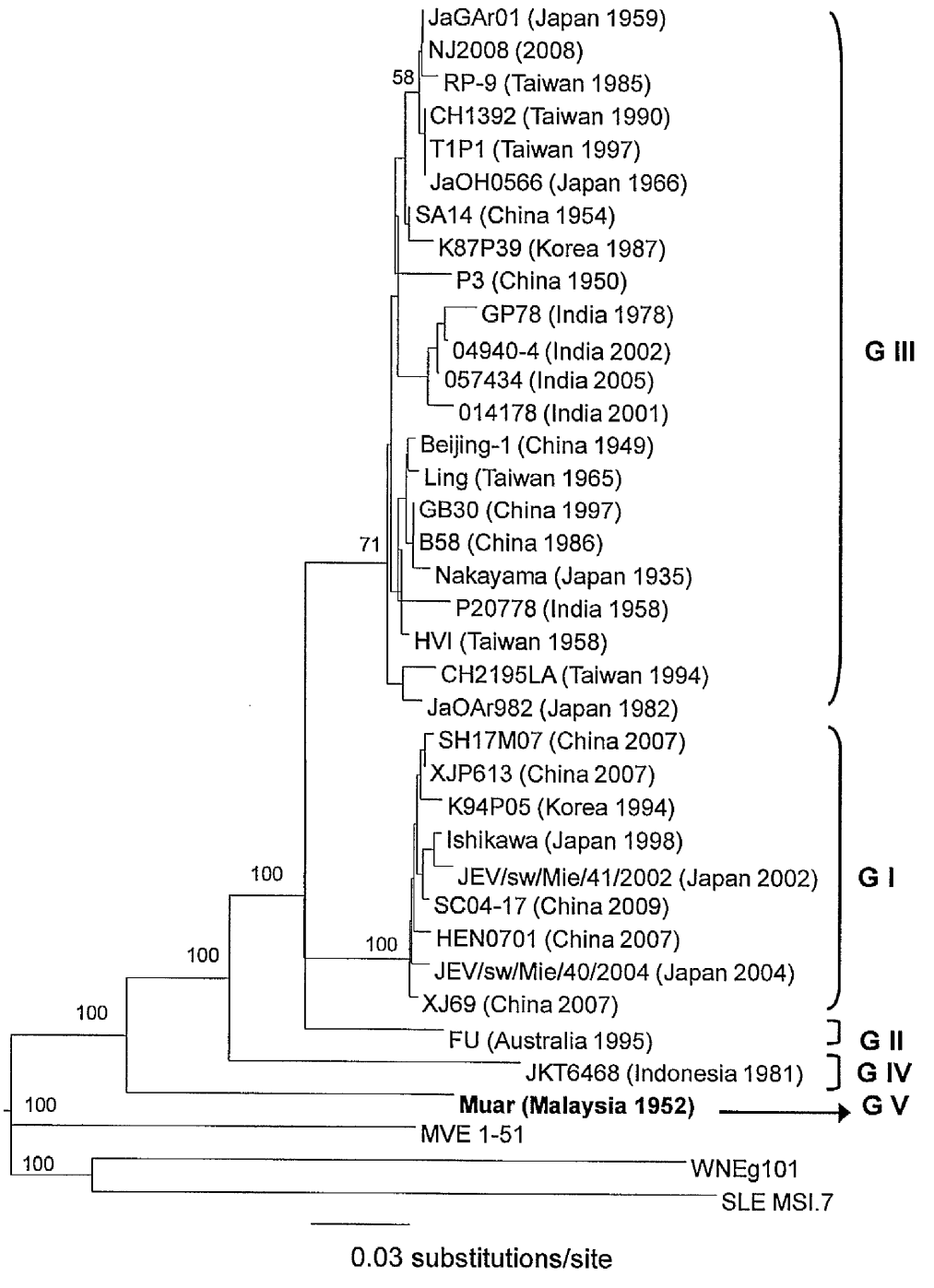


Figure 3-15. A neighbour-joining phylogenetic tree of the JEV **C** genes, rooted by using a representative strain from other viruses in the JEV serogroup (MVEV, SLE and WNV). The Muar isolate is shown with an arrow as a genotype V. Genotypes are given on the right of each tree. Bootstrap support values, given as a percentage of 1,000 replicates, are shown. Country and year of isolation for each strain used are shown in brackets.

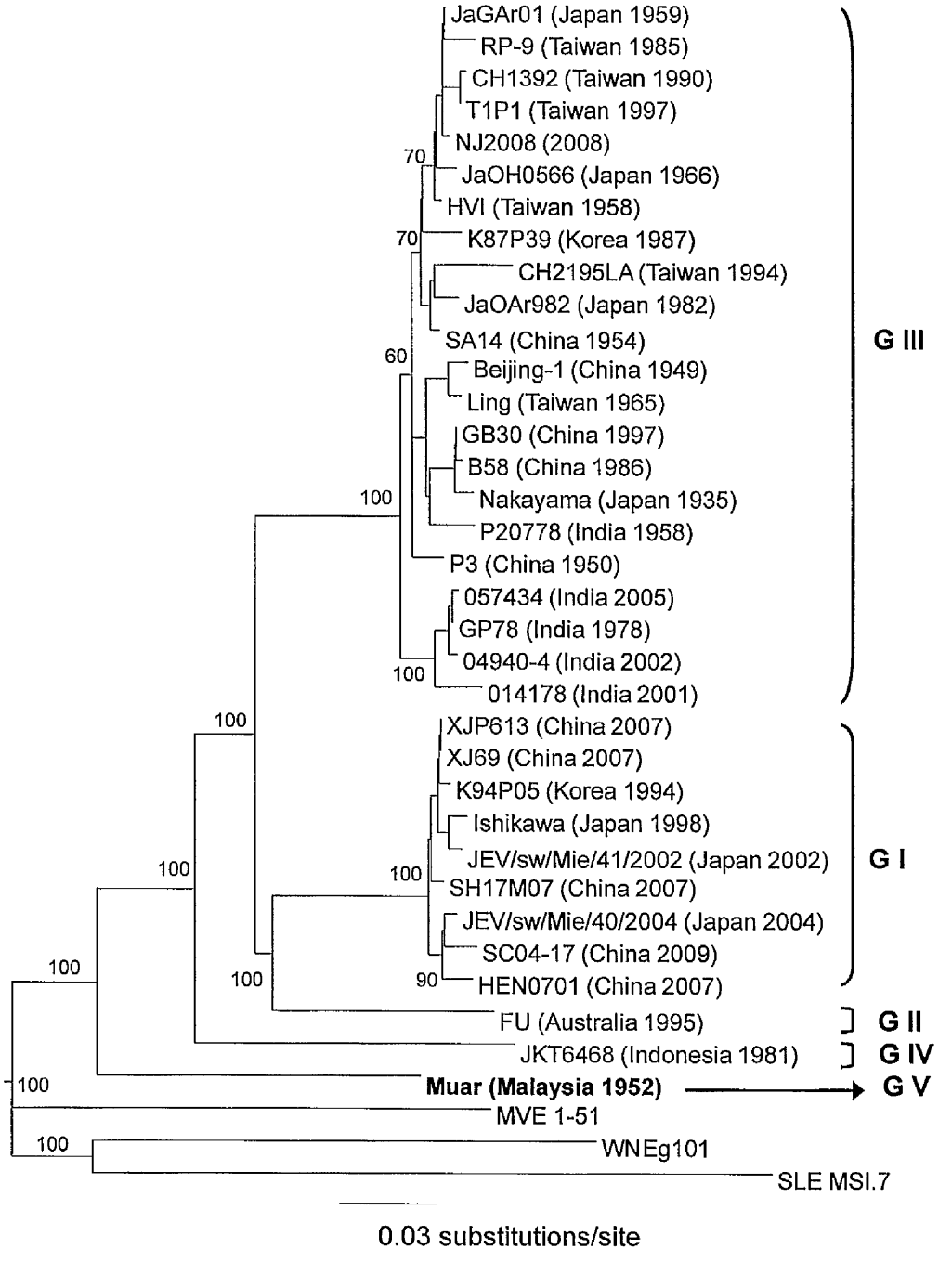


Figure 3-16. A neighbour-joining phylogenetic tree of the JEV **prM** genes, rooted by using a representative strain from other viruses in the JEV serogroup (MVEV, SLE and WNV). The Muar isolate is shown with an arrow as a genotype V. Genotypes are given on the right of each tree. Bootstrap support values, given as a percentage of 1,000 replicates, are shown. Country and year of isolation for each strain used are shown in brackets.

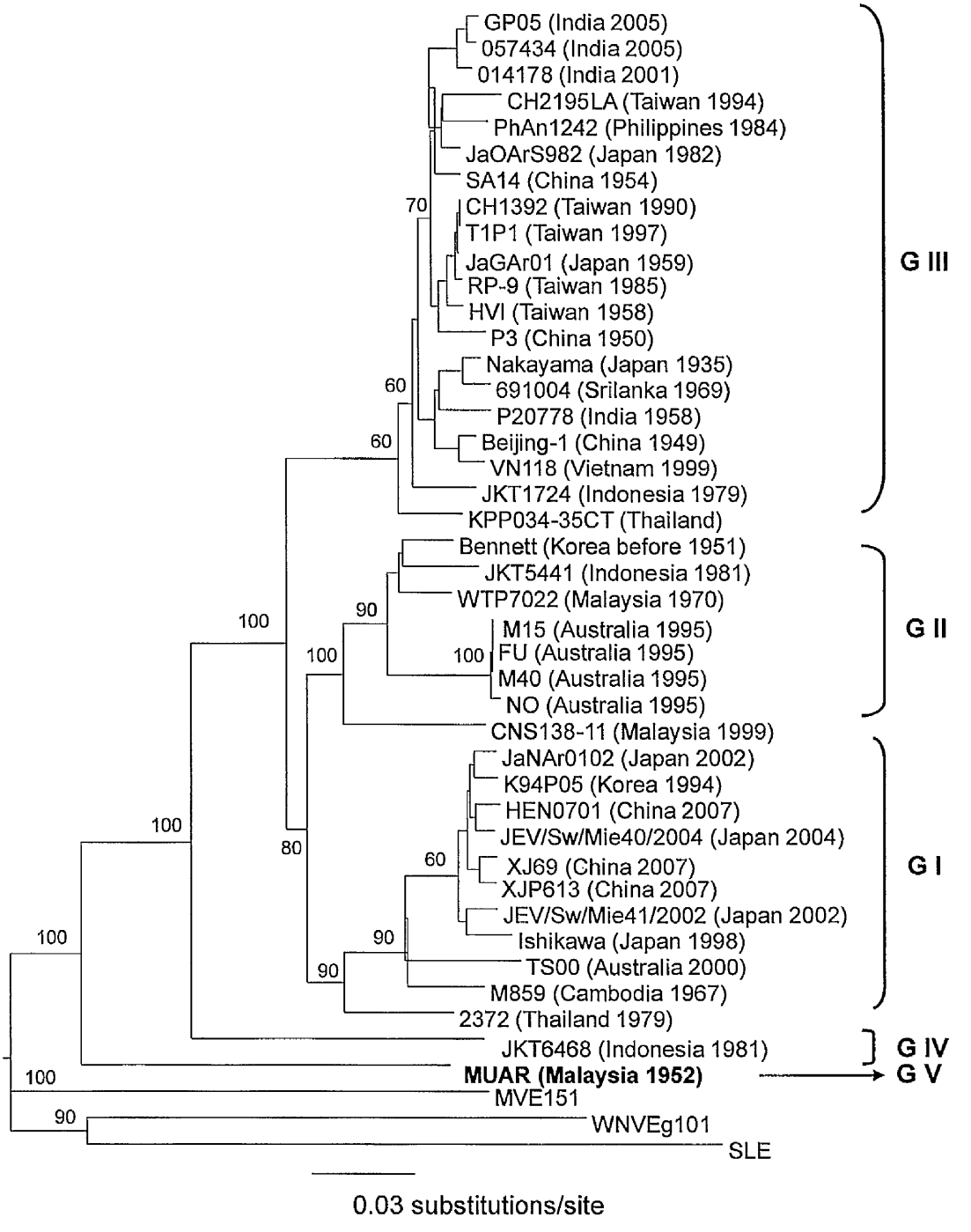


Figure 3-17. A neighbour-joining phylogenetic tree of the JEV **E** genes, rooted by using a representative strain from other viruses in the JEV serogroup (MVEV, SLE and WNV). The Muar isolate is shown with an arrow as a genotype V. Genotypes are given on the right of each tree. Bootstrap support values, given as a percentage of 1,000 replicates, are shown. Country and year of isolation for each strain used are shown in brackets.

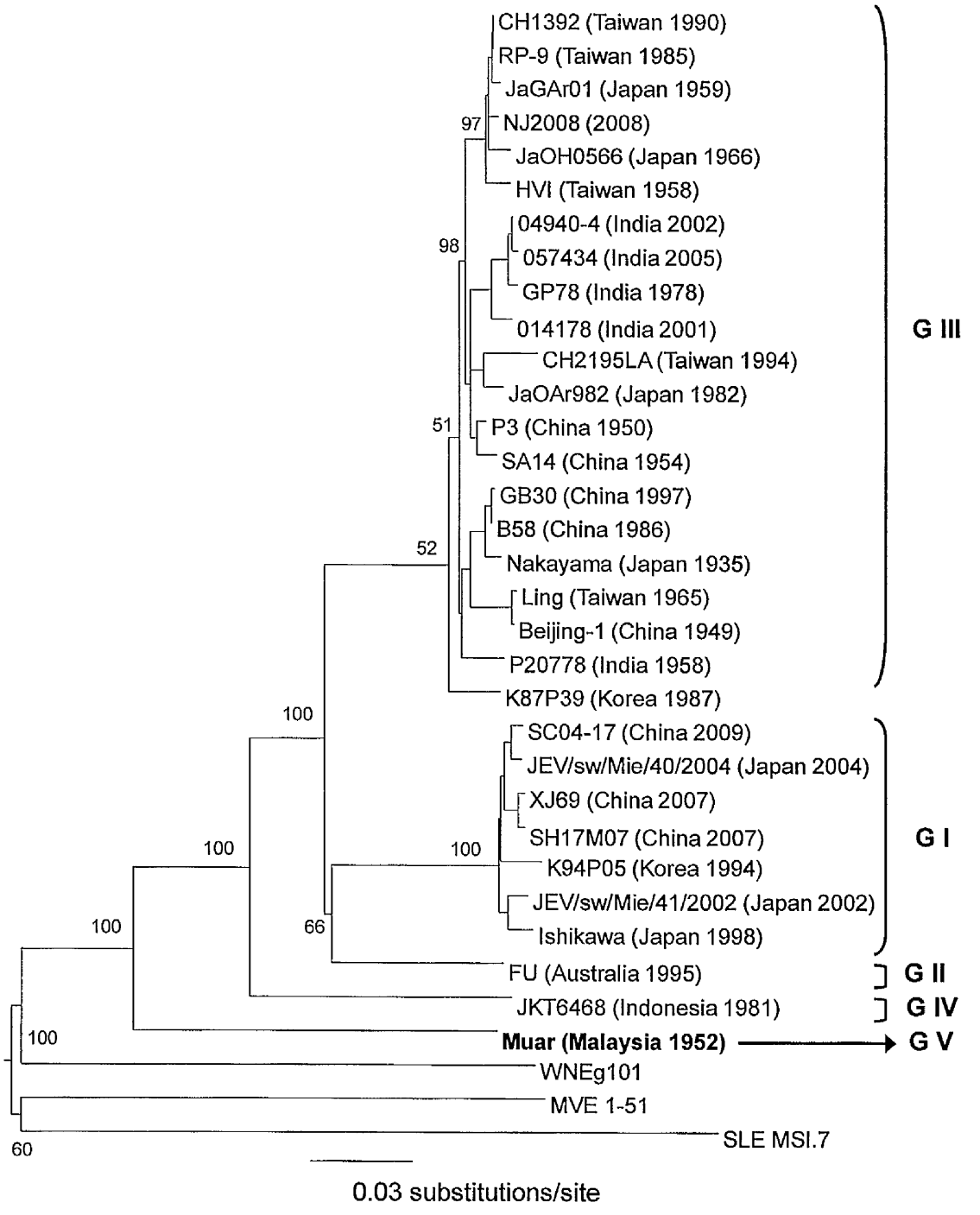


Figure 3-18. A neighbour-joining phylogenetic tree of the JEV **NS1** genes, rooted by using a representative strain from other viruses in the JEV serogroup (MVEV, SLE and WNV). The Muar isolate is shown with an arrow as a genotype V. Genotypes are given on the right of each tree. Bootstrap support values, given as a percentage of 1,000 replicates, are shown. Country and year of isolation for each strain used are shown in brackets.

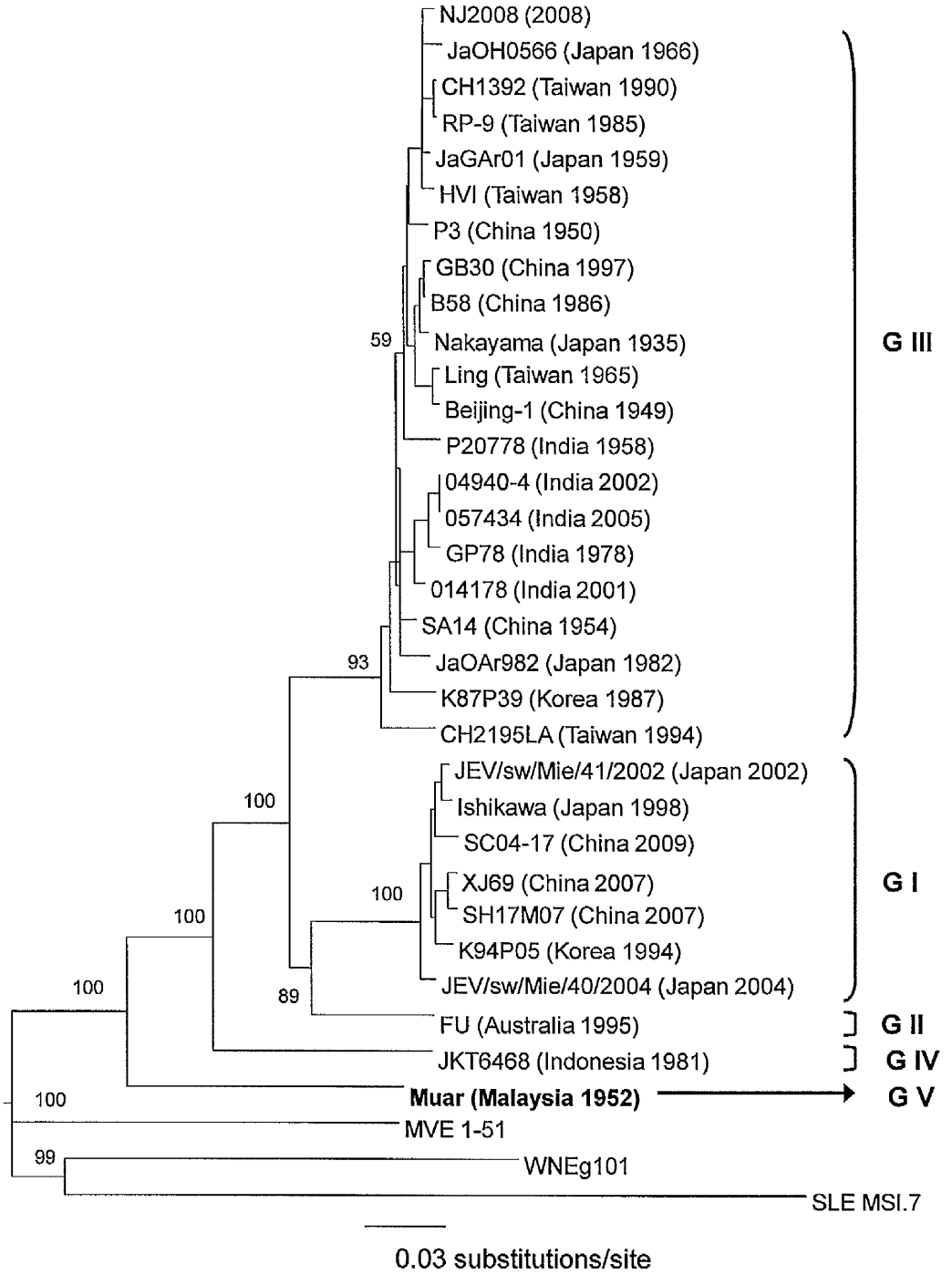


Figure 3-19. A neighbour-joining phylogenetic tree of the JEV **NS2** genes, rooted by using a representative strain from other viruses in the JEV serogroup (MVEV, SLE and WNV). The Muar isolate is shown with an arrow as a genotype V. Genotypes are given on the right of each tree. Bootstrap support values, given as a percentage of 1,000 replicates, are shown. Country and year of isolation for each strain used are shown in brackets.

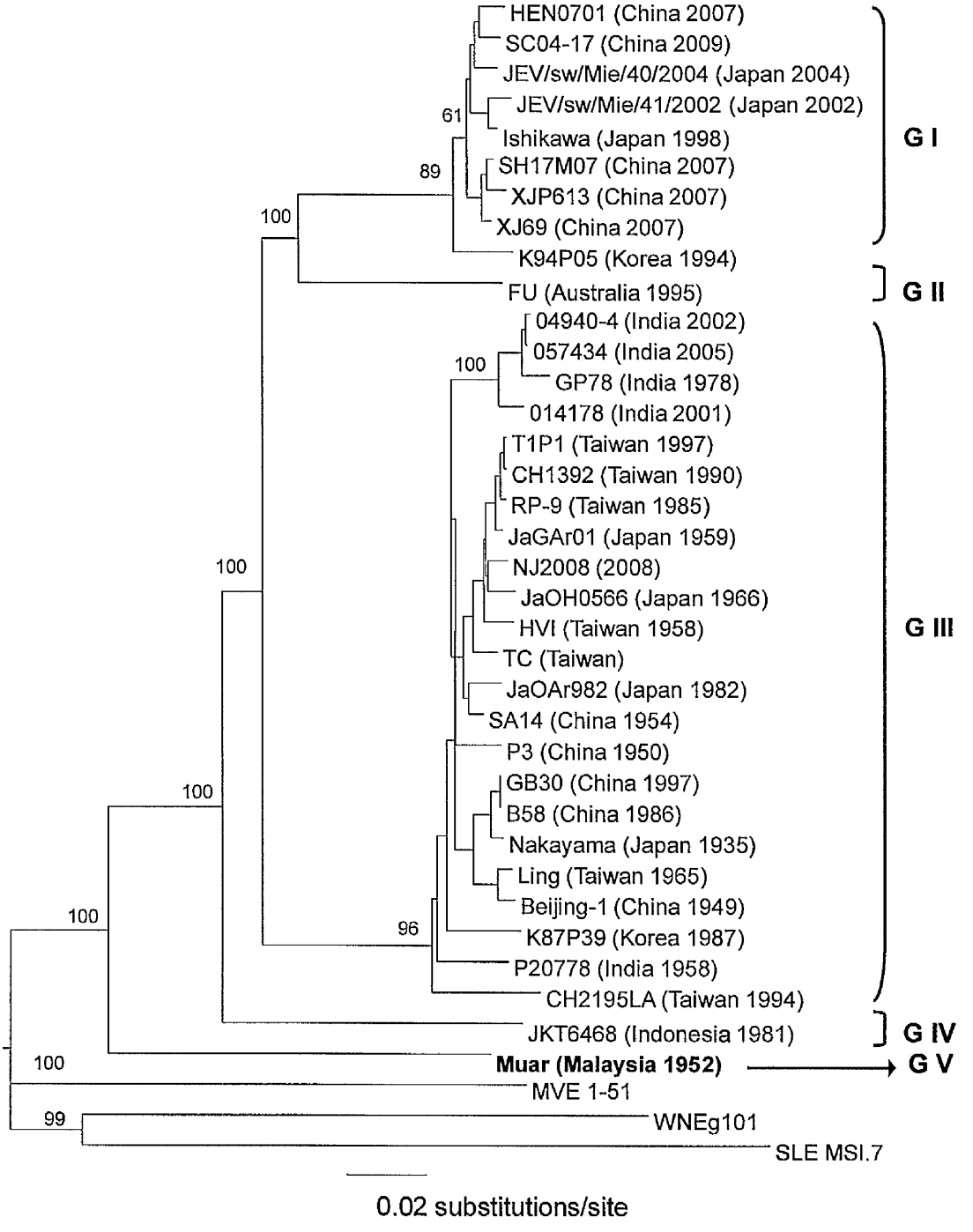


Figure 3-20. A neighbour-joining phylogenetic tree of the JEV **NS3** genes, rooted by using a representative strain from other viruses in the JEV serogroup (MVEV, SLE and WNV). The Muar isolate is shown with an arrow as a genotype V. Genotypes are given on the right of each tree. Bootstrap support values, given as a percentage of 1,000 replicates, are shown. Country and year of isolation for each strain used are shown in brackets.

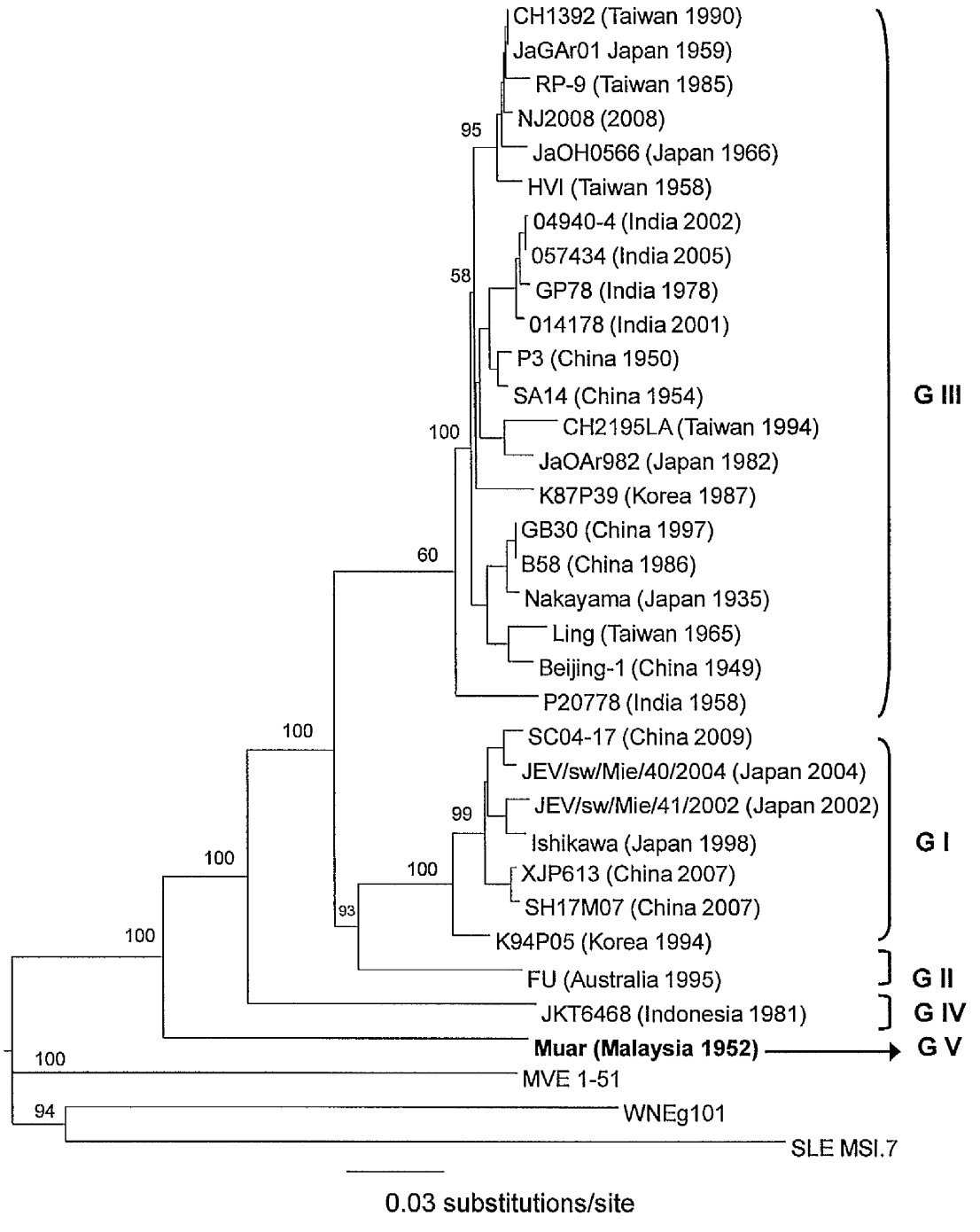


Figure 3-21. A neighbour-joining phylogenetic tree of the JEV **NS4** genes, rooted by using a representative strain from other viruses in the JEV serogroup (MVEV, SLE and WNV). The Muar isolate is shown with an arrow as a genotype V. Genotypes are given on the right of each tree. Bootstrap support values, given as a percentage of 1,000 replicates, are shown. Country and year of isolation for each strain used are shown in brackets.

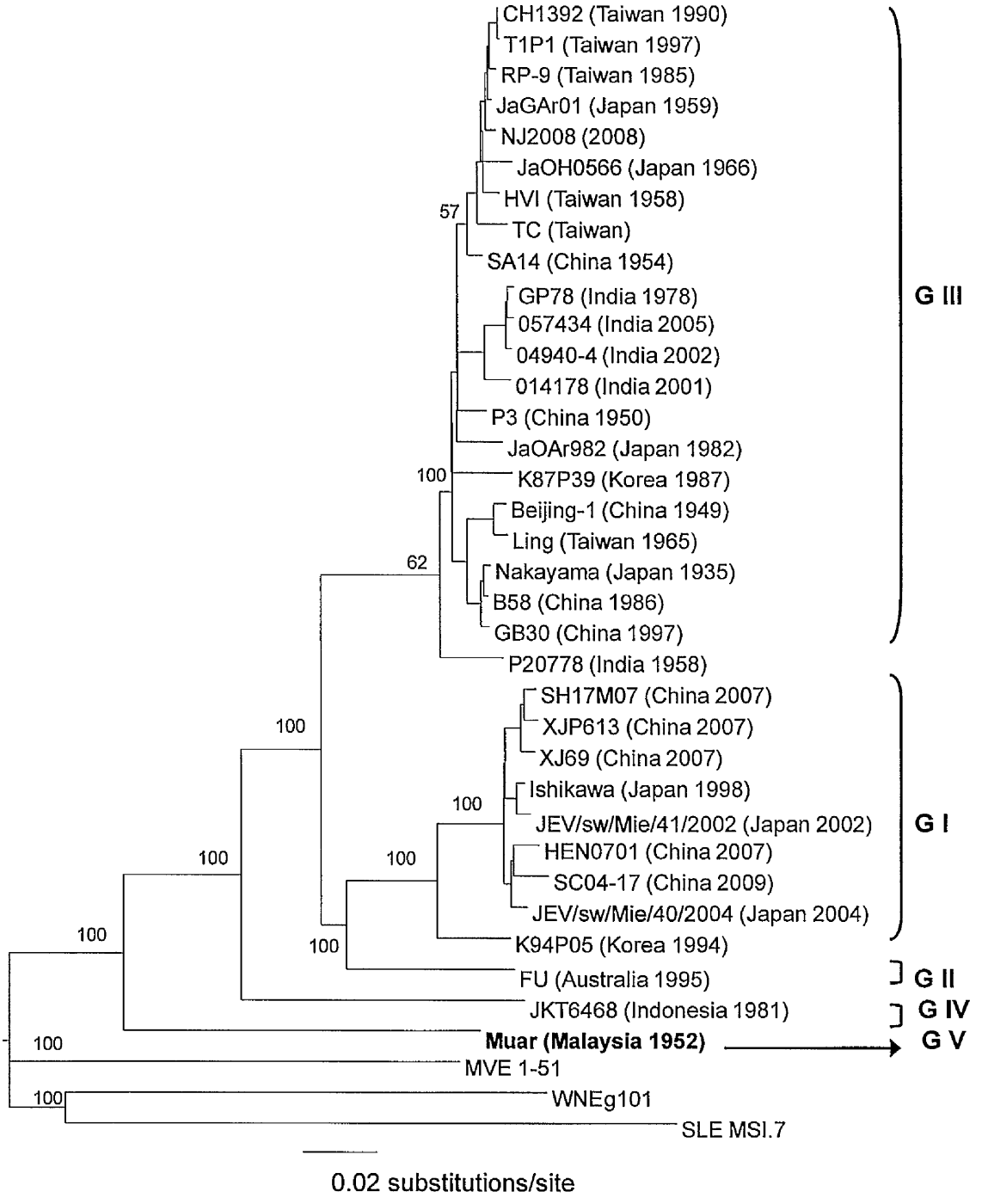


Figure 3-22. A neighbour-joining phylogenetic tree of the JEV **NS5** genes, rooted by using a representative strain from other viruses in the JEV serogroup (MVEV, SLE and WNV). The Muar isolate is shown with an arrow as a genotype V. Genotypes are given on the right of each tree. Bootstrap support values, given as a percentage of 1,000 replicates, are shown. Country and year of isolation for each strain used are shown in brackets.

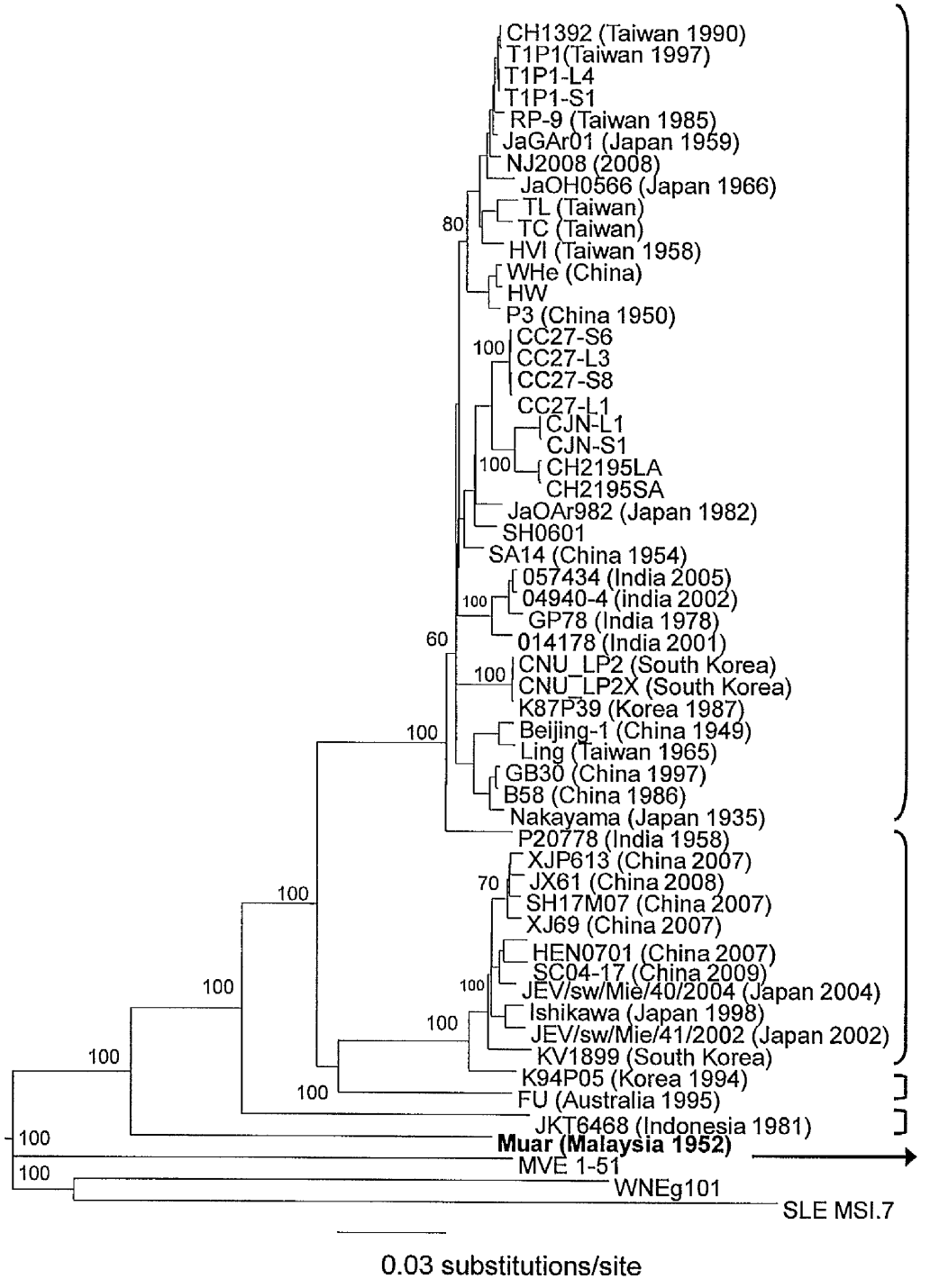


Figure 3-23. A neighbour-joining phylogenetic tree of the JEV **complete genome**, rooted by using a representative strain from other viruses in the JEV serogroup (MVEV, SLE and WNV). The Muar isolate is shown with an arrow as a genotype V. Genotypes are given on the right of each tree. Bootstrap support values, given as a percentage of 1,000 replicates, are shown. Country and year of isolation for each strain used are shown in brackets.

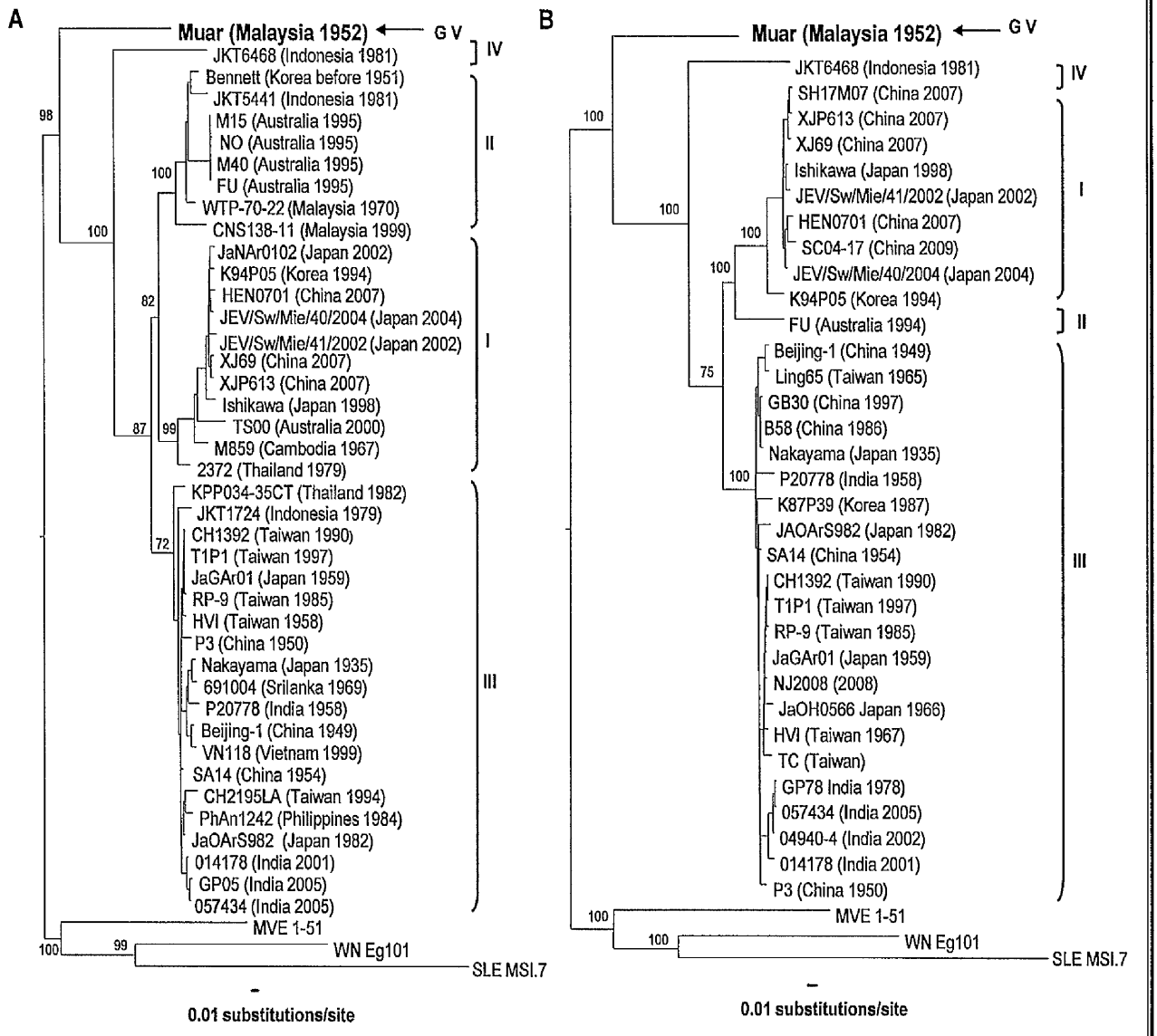


Figure 3-24. Maximum likelihood phylogenetic trees of the (A) envelope genes and (B) NS5/ 3′ NCR genes, rooted by using a representative strain from other viruses in the JEV serogroup (MVE, Murray Valley encephalitis; SLE; St. Louis encephalitis; WN, West Nile). The Muar isolate is shown with an arrow as a Genotype V. Genotypes are given on the right of each tree. Bootstrap support values, given as a percentage of 1,000 replicates, are shown.

### 3.3.6 Phylogenetic Analysis among JEV Genotypes and WNV Lineages

Based on the nucleotide and amino acid sequence of E gene of JEV genotypes strains and WNV lineages strains, the closely related virus to JEV within the JE serocomplex, NJ phylogenetic analysis revealed that the Muar isolate is the fifth genotype of JEV with 100% bootstrap support as shown in Figures 3-25 and 3-26.

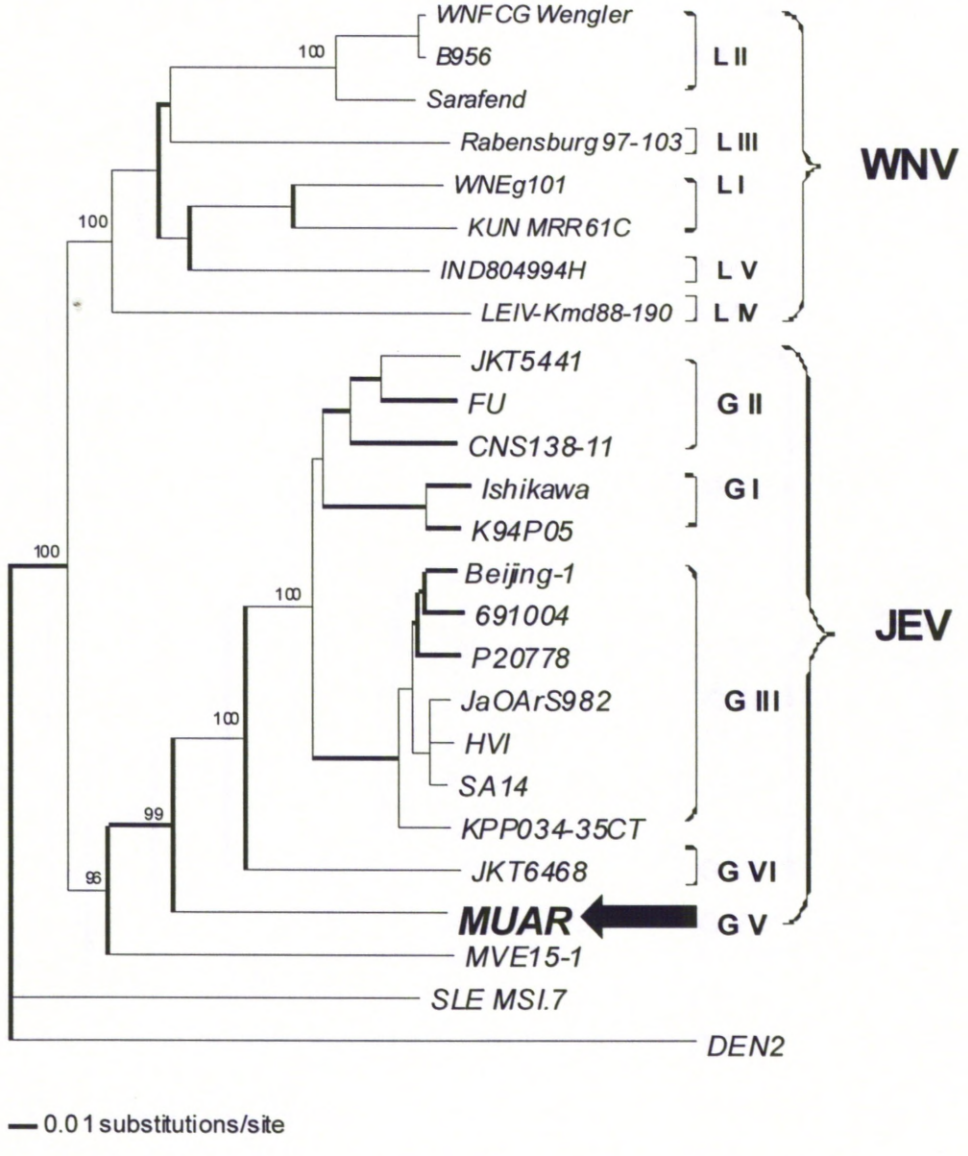


Figure 3-25. A neighbour-joining phylogeny of the nucleotide sequence of the JEV E genes five genotypes and WNV five lineages which are given on the right of each tree, with representative strains from other viruses in the JE serogroup (MVE and SLE) and outgrouped by using Dengue-2 strain New Guinea C. The Muar virus is shown with an arrow. Bootstrap support values, given as a percentage of 1,000 replicates, are shown.

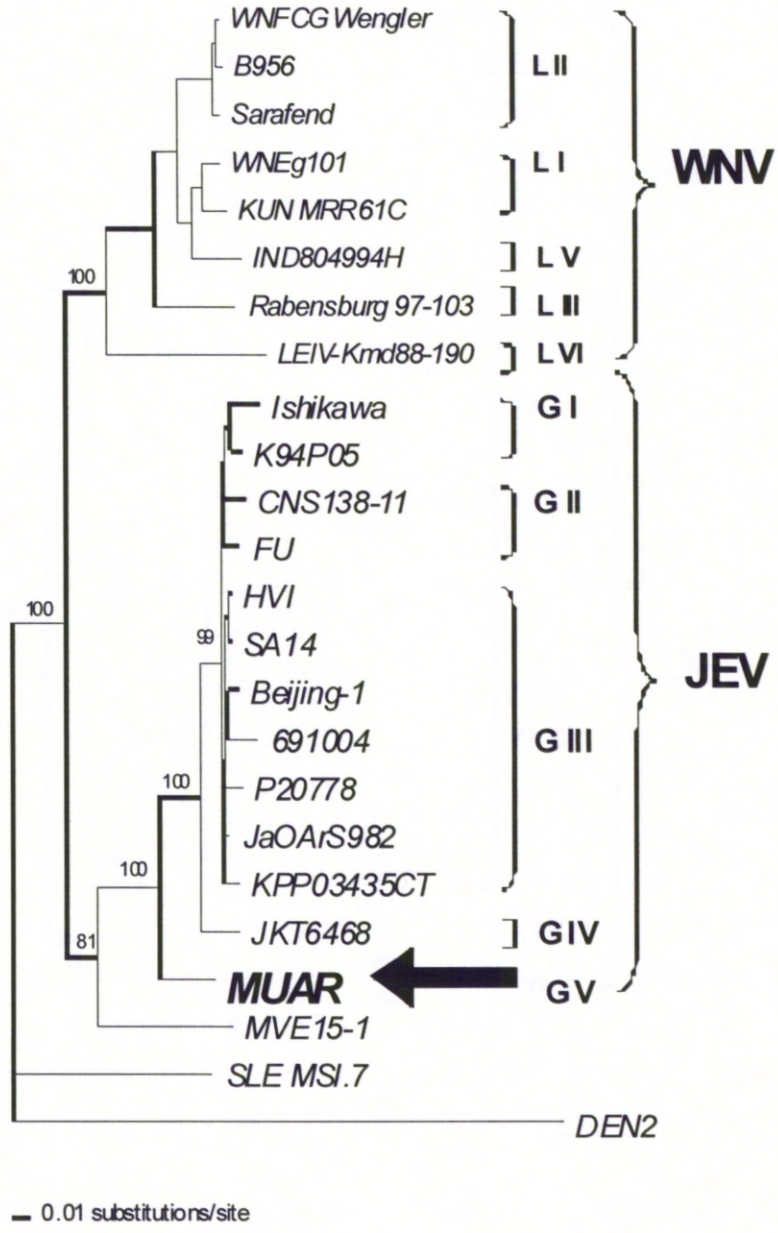


Figure 3-26. A neighbour-joining phylogeny of the amino acid sequence of the JEV E glycoprotein five genotypes and WNV five lineages which are given on the right of each tree, with representative strains from other viruses in the JE serogroup (MVE and SLE) and outgrouped by using Dengue-2 strain New Guinea C. The Muar virus is shown with an arrow. Bootstrap support values, given as a percentage of 1,000 replicates, are shown.

## 3.4 DISCUSSION

Genetic data is publicly accessible for several JE strains (more than 400 entries in NCBI GenBank), with the notable exception of the Muar strain, with 20 complete genomes for viruses from China, Japan, Korea, India, Taiwan, and Australia (Holbrook and Barrett, 2002b). The automation of nucleotide sequencing and improvements in PCR technology to make amplification more accurate and faster (e.g. longer PCR products with high fidelity) facilitate the sequencing of the complete Muar genome.

The Muar strain sequencing revealed that it has the least similarity among other JEV strains, with only (78.8 - 79.8%) homology for the nucleotides and (90.8 - 91.5%) homology for the amino acids. This indicated that Muar strain is the most structurally different from other JEV strains, in agreement with reactivities results using anti-Muar MAbs (Hasegawa, 1982; Hasegawa et al., 1995). These data are also a more direct correlate to results found in serological experiments as these studies are measuring viral structure directly.

Hasegawa et al. (1994) published the sequence of the three structural genes (C, prM and E) of the Muar isolate (sequence has not been submitted to GenBank), the number of nucleotide and amino acid differences between my Muar sequence and that generated by Hasegawa et al. (1994) varied greatly (6.7%) and (5%) respectively; the difference in the nucleotide sequence and amino acid sequences of the structural genes of the two Muar strains is given as appendix C and D repectively. Despite this great differences, phylogenetic analyses based on the nucleotide and amino acid sequences of the Muar strain, sequenced by Hasegawa et al. (1994), revealed that it is still representing the fifth genotype of JEV (dat are not presented here). However, the reasons for these differences are difficult to explain. Some differences are to be expected due to variability arising during growth of the RNA viruses in culture and PCR steps. Some differences may also be due to the differences in sequencing strategies; Hasegawa et al. (1994) used manual sequencing, whereas in this study modern automated sequencing protocols were used. Although the passage history of the Muar strain used in this study is unknown, but it is known for the Muar strain used by Hasegawa et al. (1994) as the virus was passged 24 times in suckling mouse brain and only one time in C6/36 cells since its isolation from the human brain in 1952. I was not able to get the same Muar strain which was sequenced partially by Hasegawa et al. (1994).

Phylogenetic relationship among the Muar isolate and the other JEV strains based on the complete genome, 5' and 3' NCRs, C, prM, E, NS1, NS2, NS3, NS4 and NS5 genes showed that the Muar strain represents the missing fifth genotype of JEV using different types of phylogenetic analyses; MP, NJ and ML with high bootstrap values.

The 5' NCR of JEV including the Muar strain is relatively short (95 bases in length) and exhibits high sequence identity among JEV strains, while the 3' NCR is usually longer but demonstrates extensive heterogeneity in size and sequence between different JEV strains because of long deletions, insertions, sequence repeats within the proximal part of the 3' NCR (Proutski et al., 1997). However, at the same time, there is high sequence identity at the distal part of the 3' NCR among different JEV strains as demonstrated in Figure 3-9.

Furthermore, alignment of the 5' and 3' NCR terminal sequence of the four flaviviruses which are belonging to the same serological group; JEV, MVEV, WNV and SLEV showed significant homology; the 5' terminal two residues and the 3' terminal ten residues (5' AG and CACAGGATCT 3') were identical as shown in Figure 3-10. This high identity suggests that the 3' NCR sequence may play an important role for viral proliferation (Sumiyoshi et al., 1987).

In addition to the 5' and 3' terminal primary sequence homology among JEV five genotypes, similarities in the secondary structures of the 3' NCR of JEV genotypes III and V were also observed as shown in figure 3-11. Another secondary structure of the 5' and 3' NCRs was also postulated, shown in Figure 3-12 as it has been suggested that JEV genome ends may stick to each other (Sumiyoshi et al., 1987).

WNV is a closely related flavivirus to JEV within JE serocomplex. Based on phylogenetic analyses using WNV E gene revealed that WNV could be classified into five distinct groups that differed from each other by 20–26% (Bondre et al., 2007). Phylogenetic analysis using WNV strains based on the nucleotide and amino acid sequence of E gene revealed that the Muar strain represents the fifth genotype of JEV as it is different from other JEV strains by 22.1-23.8% nucleotide difference.

Of the JEV genotypes, genotype V, the Muar strain was the basal group in almost all trees, diverging at the deepest node with 100% bootstrap support, suggesting that it represents the most ancient lineage, which branched off before genotypes I, II, III and IV.

In conclusion, I sequenced the entire genome of the Muar strain of JEV. Rigrous phylogenetic analysis based on multiple regions of the genome and distance and ML methods of phylogeny all strongly suggest that JEV has five genotypes and the Muar strain belongs to a separate genotype (genotype V).

# CHAPTER 4: EVOLUTIONARY HISTORY OF THE MUAR STRAIN OF JEV

## 4.1 INTRODUCTION

Encephalitis epidemics were repoted in Japan from the 1870s onwards. JEV was first isolated in 1935 and has subsequently been spread across Asian countries (Solomon *et al.* 2003).

The origins of JEV are uncertain, but phylogenetic analyses with other flaviviruses suggest it evolved from an African ancestral virus, perhaps as recently as a few centuries ago (Gould, 2002).

Natural selection is affecting all evolving populations. Positive selection increases the frequency of alleles that confer a fitness advantage relative to the rest of the population, or increases its genetic diversity, and negative selection removes those alleles that are deleterious.

An understanding of nucleotide substitution rates is very important in the field of molecular evolution. Partitioning the total substitution rate into synonymous (silent) and nonsynonymous (amino acid-altering) components is one of the primary objectives of evolutionary studies involving coding regions (Muse and Gaut, 1994).

Comparison of relative fixation rates of synononymous and nonsynonymous mutations provides a method for understanding the mechanisms of molecular sequence evolution. The nonsynonymous/synonymous rate ratio ($\omega = dN/dS$) is an important indicator of selective pressure at the protein level, with $\omega = 1$ meaning neutral mutations, $\omega < 1$ purifying selection, and $\omega > 1$ diversifying positive selection. Amino acid sites in a protein are expected to be under different selective pressures and have different underlying $\omega$ ratios (Yang et al., 2000).

Codon-based models of molecular evolution are able to identify the signature of selection from alignments of homologous sequences by estimating the relative rates of synonymous (*dS*) and nonsynonymous substitutions (*dN*) (Pond & Frost, 2005).

Recent studies have highlighted the potential role of selection in the ongoing evolution of flaviviruses. Flaviviruses, particularly vector borne flaviviruses, appear to be predominantly constrained by purifying selection (Holmes, 2003; Woelk and Holmes, 2002), but evidence is beginning to emerge that some flavivirus proteins are subject to positive selection. The ML

approach has been used to detect adaptive evolution in the four serotypes of DENV. For example, positive selection appeared to be acting primarily on the E gene of DENV, on amino acids involved in immune recognition, cell receptor binding, and membrane fusion (Twiddy et al., 2002). Recently, a single amino acid substitution in the NS3 protein has been identified, which confers high virulence on otherwise low virulence strains of WNV (Brault et al., 2006).

To date, many phylogenetic analyses suggest the presence of strong purifying (negative) selection on arboviruses in nature, supporting strong selective constraints on flavivirus evolution (Weaver and Vasilakis, 2009). The global $d$N/$d$S of 0.05 agreed with previous estimates in JEV and other flaviviruses (Tang et al., 2010b; Woelk and Holmes, 2002).

Until now, only limited data of the selection pressures acting on JEV have been available (Tang *et al.*, 2010; Yang *et al.*, 2000) but none of these have included the Muar strain in the analysis, which we have shown represents a fifth genotype of JEV, and may provide important additional information. Here, I examine the evolutionary history of JEV looking in particular at selection pressures.

To understand the role that selection plays in JEV evolution, and in order to identify the patterns of genetic change of JEV genotypes, overall and site specific selection pressures in the E genes of 66 JEV strains belonging to the five JEV genotypes were measured.

## 4.2 MATERIALS & METHODS

### 4.2.1 Evolutionary History of the Muar Isolate

A maximum clade credibility (MCC) tree based on 35 complete coding sequences of JEV was inferred using the bayesian MCMC method available in the BEAST package (version 1.5b2) (Drummond and Rambaut, 2007), thereby incorporating information on the isolates time of original sampling. This analysis utilized a relaxed molecular clock (uncorrelated lognormal) and a GTR + Gamma + Invariant model of nucleotide substitution for each codon position. All chains were run for a sufficient length to ensure convergence with 10% removed as burn-in. This analysis also allowed estimation of coalescent (divergence) times for each node on the JEV phylogeny. The degree of uncertainty in each parameter estimate is

provided by the 95% highest posterior density (HPD) values, while posterior probability values provide an assessment of the degree of support for each node on the tree.

The evolutionary rate of the complete genome amongst representative strains of genotypes I and III, and for the E gene amongst strains of genotypes I, II and III was estimated. Such analysis was not possible between the strains of genotypes IV and V as there is only one representative strain published on GenBank.

### 4.2.2 Selection Pressures between JEV Genotypes

Overall and site specific selection pressures in the E genes of 66 JEV strains belonging to the five JEV genotypes were measured as the nonsynonymous/synonymous rate ratio ($\omega = dN/dS$) using a ML method implemented by Datamonkey (Pond and Frost, 2005). Tests for positive selection were performed using three methods; single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), and internal FEL (IFEL) (http://www.datamonkey.org).

## 4.3 RESULTS

### 4.3.1 Evolutionary History of the Muar

Molecular clock analysis of 35 complete coding sequences of JEV, supported by high posterior probability values, confirmed that Muar belongs to a fifth JEV genotype and represents the oldest linage of JEV, which last shared a common ancestor with other genotypes approximately 449.6 years ago (95% highest posterior density interval "HPD"= 373.9 – 499.9). Genotype IV diverged from other genotypes approximately 274 (211.6 – 317.8) years ago, genotype II approximately 189 (116.8 – 237.6) years ago, genotype III approximately 158 (111.3 – 183.7) years ago, and most recently Genotype I approximately 110 (78.5 – 167.8) years ago as shown in Figure 4-1.

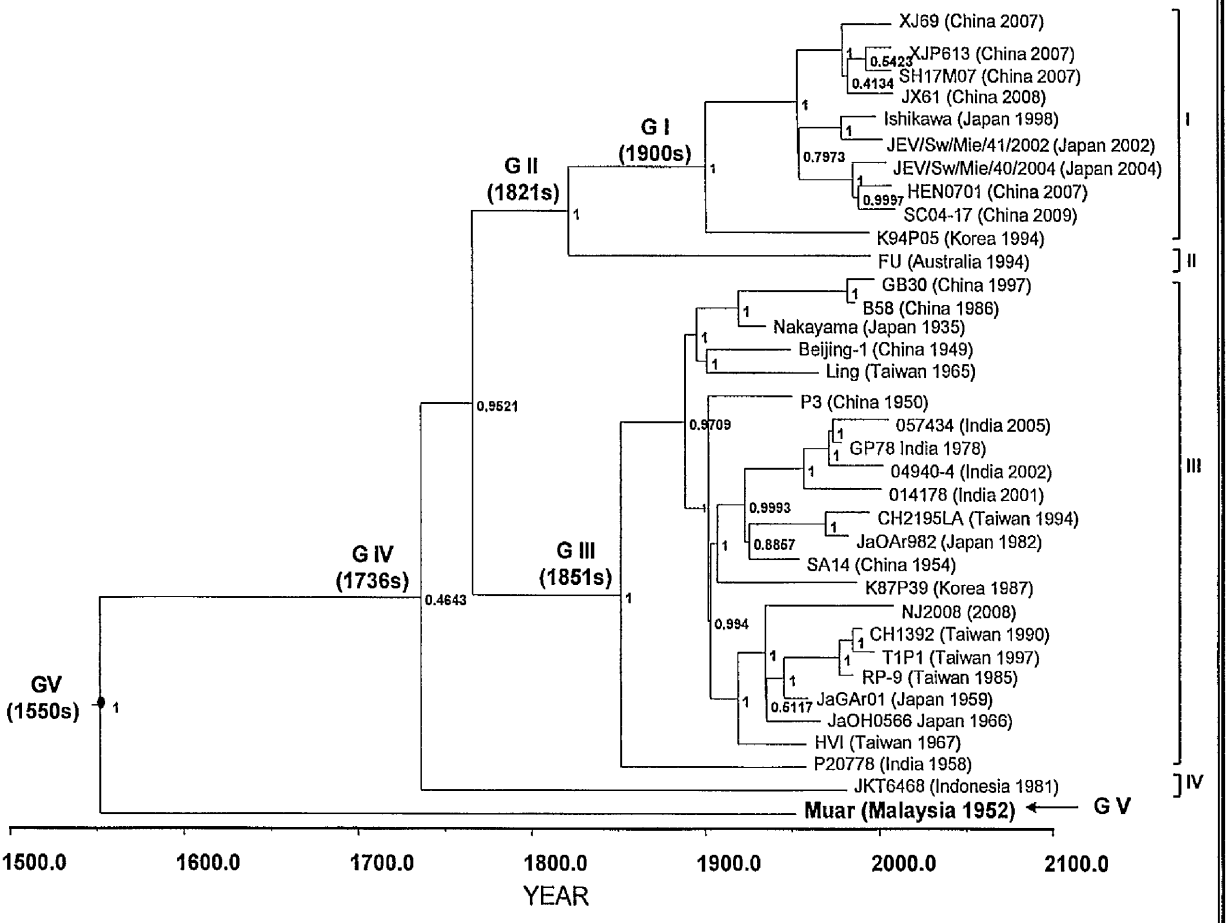


Figure 4-1. Maximum clade credibility (MCC) phylogeny for 35 complete coding sequences of JEV genomes. Horizontal branches are drawn to a scale of estimated year of divergence with tip times reflecting sampling date (year). The tree is automatically rooted due to the assumption of a molecular clock. The divergence times of genotype nodes are shown. High posterior probability values are shown beside the nodes. The filled black circle identifies the most recent common ancestor of JEV. The Muar isolate, the oldest linage, is shown with arrow as a Genotype V.

Evolutionary analysis of full-length JEV genomes revealed that the mean evolutionary rate of JEV isolates is $4.35 \times 10^{-4}$ nucleotides substitutions per site per year. At the genotype level, the mean evolutionary rate of genotypes I and III complete genomes was $3.029 \times 10^{-4}$ and $6.029 \times 10^{-4}$ nucleotides substitutions per site per year respectively. Comparing the mean evolutionary rate of each genotype separately using E genes showed that the mean evolutionary rate between genotypes I, II and III was $1.14 \times 10^{-3}$, $1.97 \times 10^{-3}$ and $2.69 \times 10^{-4}$ nucleotides substitutions per site per year respectively. 95% lower and upper HPD values are shown in (Table 4-1).

Table 4-1. Rates of nucleotide substitutions per site for JEV complete genomes and E genes.

| JEV genotype | Mean no. of nucleotide substitutions/site/year | 95% HPD* | |
|---|---|---|---|
| | | Lower | Upper |
| GI-V complete genomes | $4.35 \times 10^{-4}$ | $3.4906 \times 10^{-4}$ | $5.303 \times 10^{-4}$ |
| GI complete genomes | $3.029 \times 10^{-4}$ | $2.0884 \times 10^{-8}$ | $8.6999 \times 10^{-4}$ |
| GIII complete genomes | $6.029 \times 10^{-4}$ | $7.0492 \times 10^{-8}$ | $1.4093 \times 10^{-4}$ |
| GI envelope genes | $1.14 \times 10^{-3}$ | $2.3 \times 10^{-4}$ | $2.2 \times 10^{-3}$ |
| GII envelope genes | $1.97 \times 10^{-3}$ | $2.8 \times 10^{-4}$ | $3.95 \times 10^{-3}$ |
| GIII envelope genes | $2.69 \times 10^{-4}$ | $1.09 \times 10^{-4}$ | $4.20 \times 10^{-4}$ |

*HPD= Highest Posterior Density interval.

### 4.3.2 Selection Pressures between the Five JEV Genotypes

There was no evidence for positive selection between the JEV five genotypes strains and different selection pressures models did not have any category with $\omega > 1$. The average $\omega$ ratio (dN/dS) over the entire E gene sites was estimated to be ~ 0.05, suggestive of predominantly purifying selection.

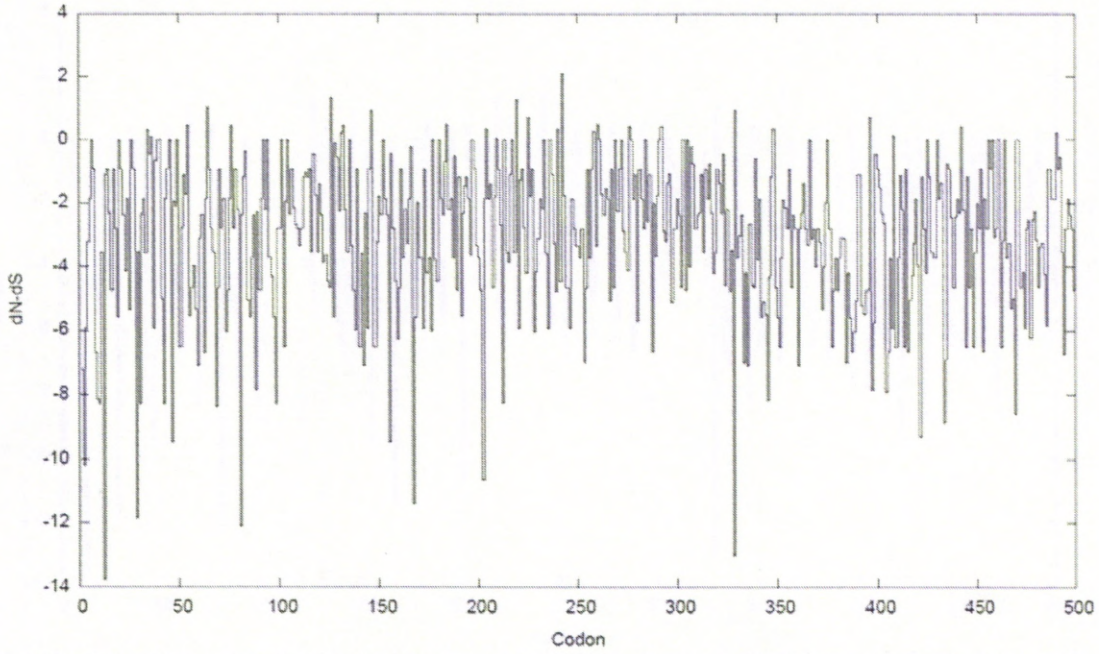


Figure 4-2. Codon-based analysis of selection pressures on E gene of JEV strains using the sinlge likelihood ancestor counting method (SLAC, Datamonkey). The Figure shows a plot of *d*N/*d*S values for each predicted codon of the consensus sequences.

*dN-dS is the logarithm of the ratio of ω (*d*N/*d*S).

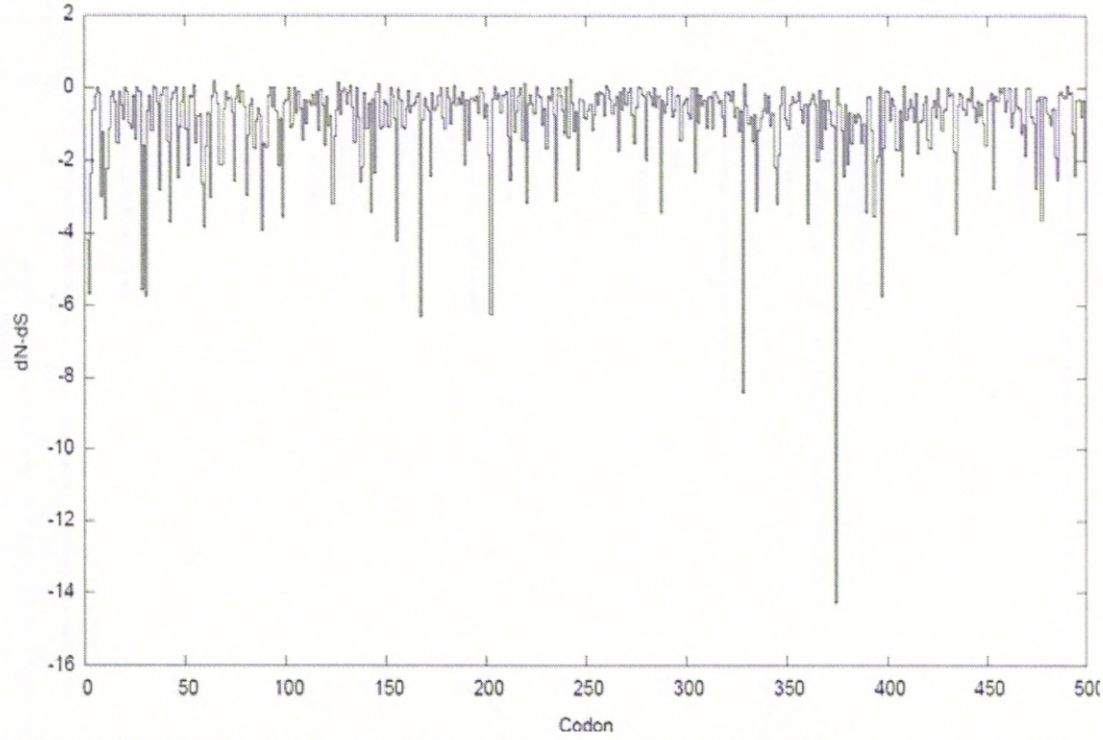


Figure 4-3. Codon-based analysis of selection pressures on E gene of JEV strains using the fixed effects likelihood method (FEL, Datamonkey). The Figure shows a plot of *d*N/*d*S values for each predicted codon of the consensus sequences.

*dN-dS is the logarithm of the ratio of ω (*d*N/*d*S).

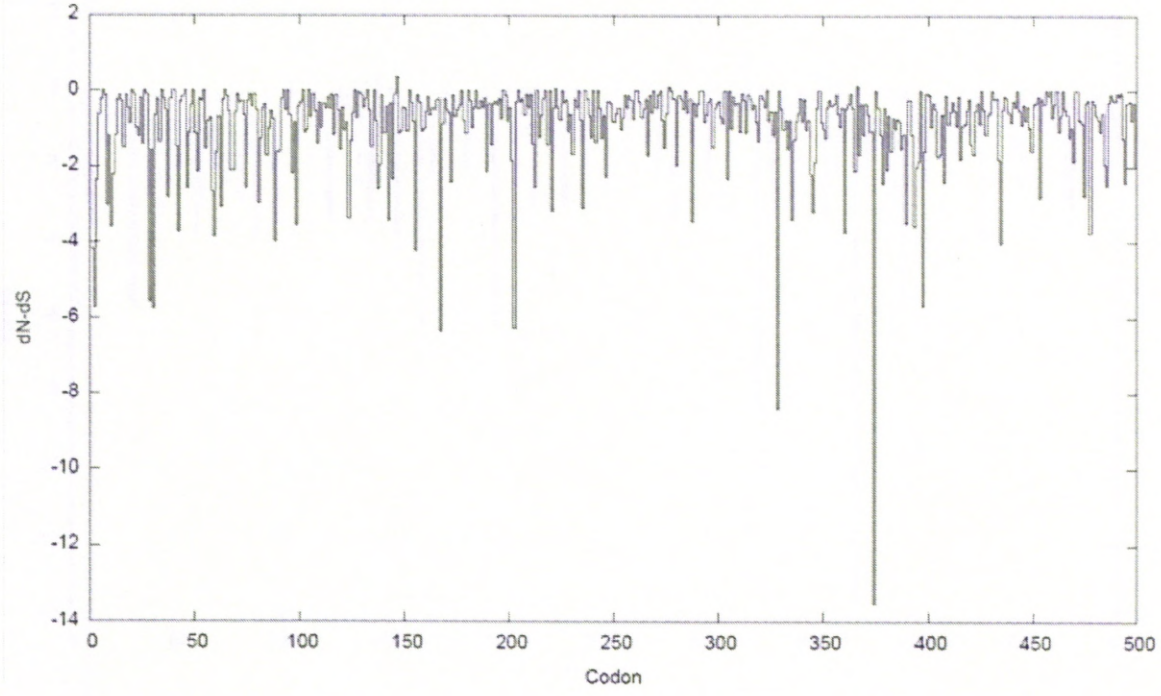


Figure 4-4. Codon-based analysis of selection pressures on E gene of JEV strains using the internal fixed effects likelihood method (IFEL, Datamonkey). The Figure shows a plot of *d*N/*d*S values for each predicted codon of the consensus sequences.

*dN-dS is the logarithm of the ratio of ω (*d*N/*d*S).

### 4.3.3 Pattern of the Geographical Distribution of JEV Isolates

NJ tree was constructed based on the nucleotide sequence of the JEV E genes available at GenBank. JEV strains were isolated from different geographical regions and from different hosts (mosquito, pig or human brain), These geographical and host characteristics were generally not reflected in the phylogeny, rather the strains largely appear mixed up (Figures 4-5 and 4-6). However, JEV Indian strains could be clustered as all of them are belonging to genotypes III and JEV strains isolated from different hosts in Taiwan are also belonging to genotypes III. Almost all of JEV Australian strains are belonging to genotype I, while Japanese and Chinese isolates are belonging to genotypes I and II.

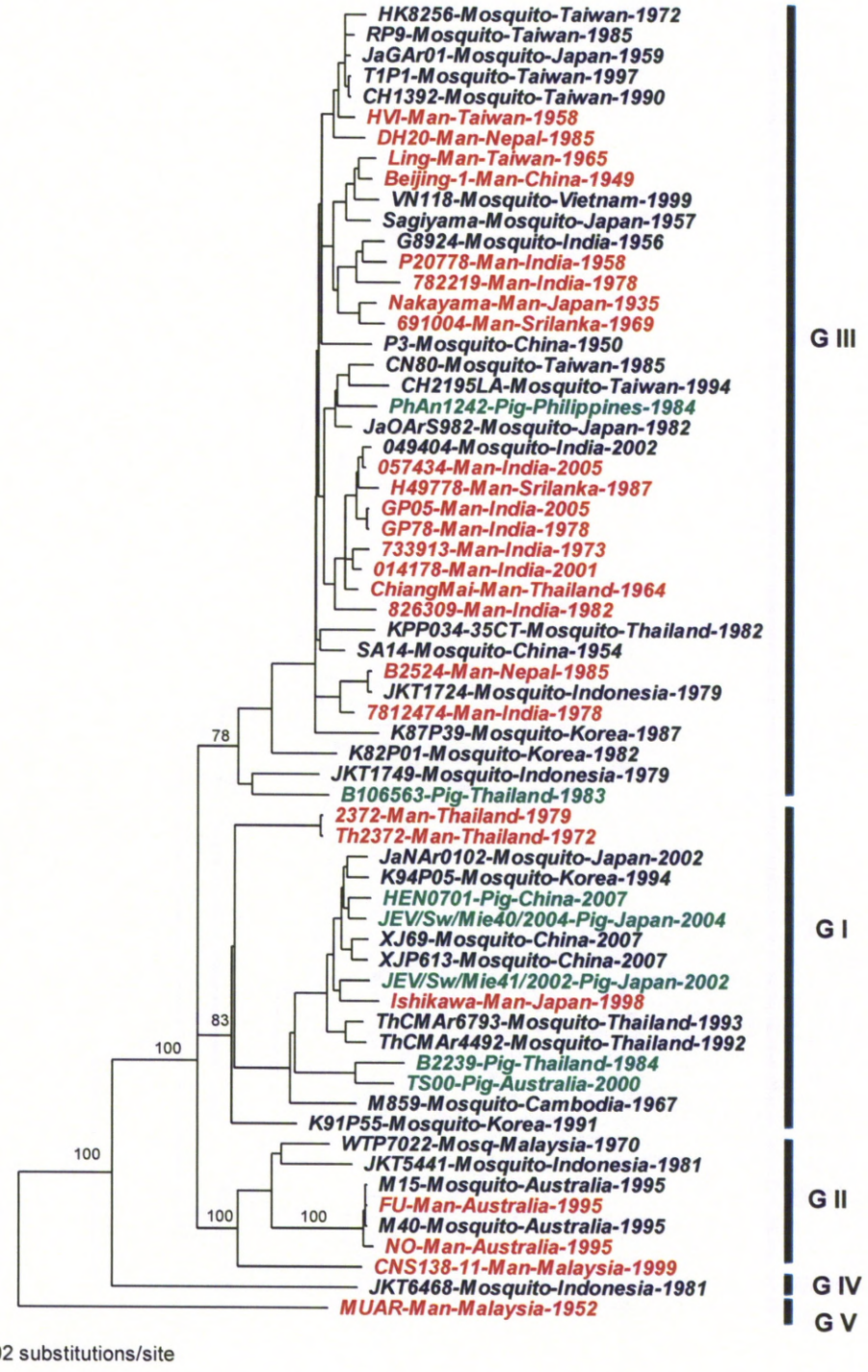


Figure 4-5. A neighbour-joining phylogenetic tree of JEV E genes. Genotypes are given on the right of each tree. Each strain has the year of isolation, the country and the host. **Each colour represents one host.** Bootstrap support values, given as a percentage of 1,000 replicates, are shown.

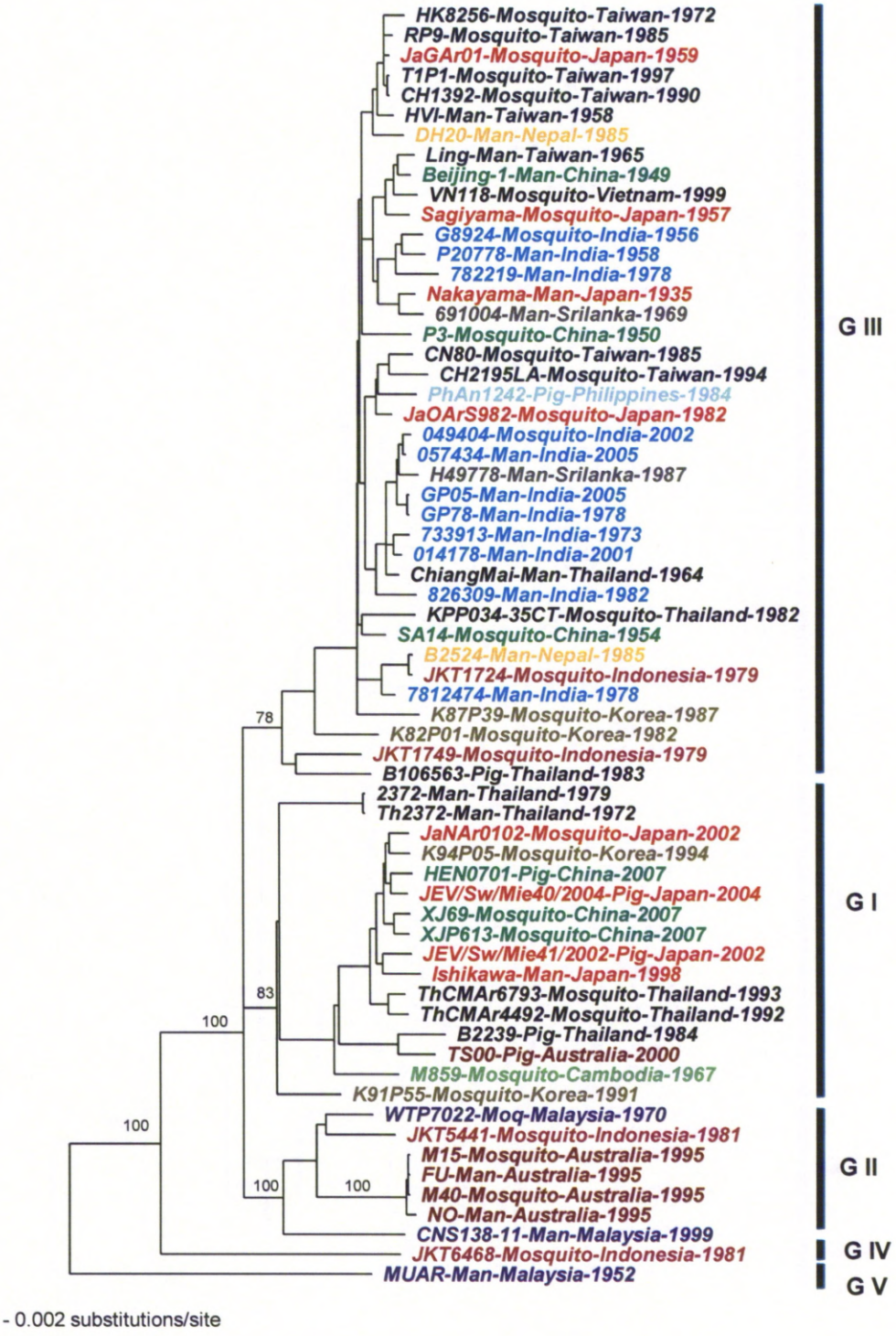


Figure 4-6. A neighbour-joining phylogenetic tree of JEV E genes. Genotypes are given on the right of each tree. Each strain has the year of isolation, the country and the host. **Each colour represents one country**. Bootstrap support values, given as a percentage of 1,000 replicates, are shown.

## 4.4 DISCUSSION

We have little idea about the origin of most of the major human pathogens. However, because JEV has recently evolved and has so rapidly spread to new areas, it has been possible to trace its geographical origin.

Here I have shown that the Muar strain represents the oldest JEV lineage, and compared it with other fully sequenced genomes. Previous studies suggest that JEV arose from its ancestral virus in the Indonesia-Malaysia region and evolved there into five genotypes; genotypes IV and V, the most divergent genotypes, remained confined to the Indonesia-Malaysia region, whereas genotypes I, II and III, the most recently evolved genotypes spread across Asia (Solomon et al., 2003b).

In this chapter, detailed examination of the geographical distribution of all known JEV isolates is consistent with the idea that JEV originated from its ancestral virus in or near Malaysia in the mid 1500s; 450 (374 - 500) years ago. Subsequently genotype IV diverged, followed next by genotype III, then genotypes I and II diverged from each other. However, although my analysis is supported by high posterior probability values, we still need to include more fully sequenced strains from each genotype, especially genotypes II, IV and V to give us more accurate idea about JEV origin and spread.

The Indonesia-Malaysia region is geographically close to Australia, where JEV's closest fully sequenced relative, MVEV, is found (van den Hurk et al., 2010). JEV originated in the Indonesia-Malaysia region from an ancestral virus common to JEV and MVEV. From this ancestral virus JEV genotypes IV and V diverged, followed by the more recent genotypes I, II and III. Whereas more recent genotypes have spread to other areas, the more divergent genotypes (IV and V) appear geographically confined to the Indonesia-Malaysia region and have not been found in any other countries.

To determine whether genotype V circulated in Malaysia or the Muar strain represented a single imported case, Malaysian strains from GenBank were analysed. There were only 4 strains from Malaysia and they belonged to genotypes II and III as showed in the previous phylogenetic analysis (Solomon et al., 2003b). Thus the Muar strain represents the only isolate of genotype V virus. However, there is no nucleotide sequence information available

on Malaysian strains isolated between 1952 and 1965, between 1965 and 1968, between 1986 and 1970 and between 1970 to 1999. Therefore, it remains unknown whether the Muar strain isolation was the result of a genotype V epidemic focus that quickly died off, or a genotype V strain that circulated in Malaysia for a period of time prior to extinction, or the Muar strain was a single imported case from another region.

The evolutionary rate of E genes for each genotype separately showed a higher evolutionary rate for the most recently evolved genotypes I and II than for genotype III. However, the number of genotypes I and II strains that have sequence data available in Genbank is less than for genotypes III. Further sequencing of strains belong to the genotypes I and II from different time intervals may affect the evolutionary rate of the E gene of genotypes I and II to be similar to genotype III.

Evolutionary analysis of full-length JEV genomes revealed that the evolutionary rate of isolates is $4.35 \times 10^{-4}$ ($3.4906 \times 10^{-4}$ to $5.303 \times 10^{-4}$) nucleotides substitutions per site per year, similar to other viruses in the family *Flaviviridae*. Previous studies using BEAST have revealed an evolutionary rate of $8.48 \times 10^{-4}$ substitutions/site/year for DENV (Weaver and Vasilakis, 2009). Additionally, evolutionary rate calculations indicate that WNV has evolved at approximately $0.85 \times 10^{-3}$ substitutions/site/year (Bertolotti *et al*, 2007). The high evolution rate of JEV genomes is consistent with that of other RNA viruses, and probably explained due to the lack of a proofreading function and error-repairing ability of viral RNA polymerases (Steinhauer and Holland, 1987), the resulting accumulated mutations providing the genomic variability necessary to drive viral evolution in the genus *Flavivirus*, including JEV.

The evolutionary analysis presented here confirms that the Muar strain of JEV is not only from the fifth genotype but represents the oldest JEV lineage and from it the other four genotypes were diverged since mid 1500s.

The E protein has been demonstrated to be a reliable phylogenetic marker for JEV (Ali et al., 1995; Mangada and Takegami, 1999). It plays an important role in tissue tropism, cell fusion and infection, virus maturation, and protection (Westaway, 1987). A more detailed phylogenetic analysis of E gene showed that JEV strains have not got a specific geographical distribution pattern. The reason for this mixing up may be because JEV is a mosquito borne

zoonotic virus which can be widely and rapidly transmitted between different geographical areas.

To improve our ability to assess the roles of selection in the evolution of JEV, the codon *d*N/*d*S ratios in the E glycoprotein of 66 JEV strains belonging to the five JEV genotypes were analysed. Many sites seemed to be under negative selection pressure, and no evidence for positive selection was found. Indeed, the overall picture obtained was that of strong purifying selection, with mean *dN/dS* value ~ 0.05 for all JEV genotypes, and in agreement with previous studies (Tang et al., 2010a). This suggests that selection pressures acting on the E gene, which might include the immune response, are unlikely to be responsible for the genotypic diversification of this virus.

In conclusion, we have shown that the Muar strain represents the oldest JEV lineage and JEV's origin is back to mid 1500s. Although E protein is subjected to strong negative pressure, but understanding the genetic changes in the E gene is an important step to study the evolution of JEV. More extensive surveillance might help us understand geographic movement and genotype shift of JEV.

# CHAPTER 5: PREDICTION OF THE THREE DIMENSIONAL STRUCUTURE OF THE ENVELOPE GLYCOPROTEIN OF THE MUAR STRAIN OF JEV

## 5.1 INTRODUCTION

JEV is an RNA-enveloped virus (Monath and Heinz, 1996). The E glycoprotein, the major antigenic determinant on flavivirus virions, mediates receptor binding and membrane fusion during virus entry. It is responsible for a number of important functions including cellular tropism, virulence, induction of neutralizing antibodies and protective immunity (Chambers et al., 1990).

Each E protein subunit is composed of three domains: domain I, which forms a $\beta$-barrel; domain II, which projects along the virus surface between the transmembrane regions of the homodimer subunits; and domain III, which maintains an Ig-like fold (Allison et al., 2001; Rey et al., 1995a). Domain III appears to be involved in receptor binding and is a major target of neutralizing antibodies. Figure 5-1 illustrates the organization of the E protein dimer as present at the surface of mature flavivirus.

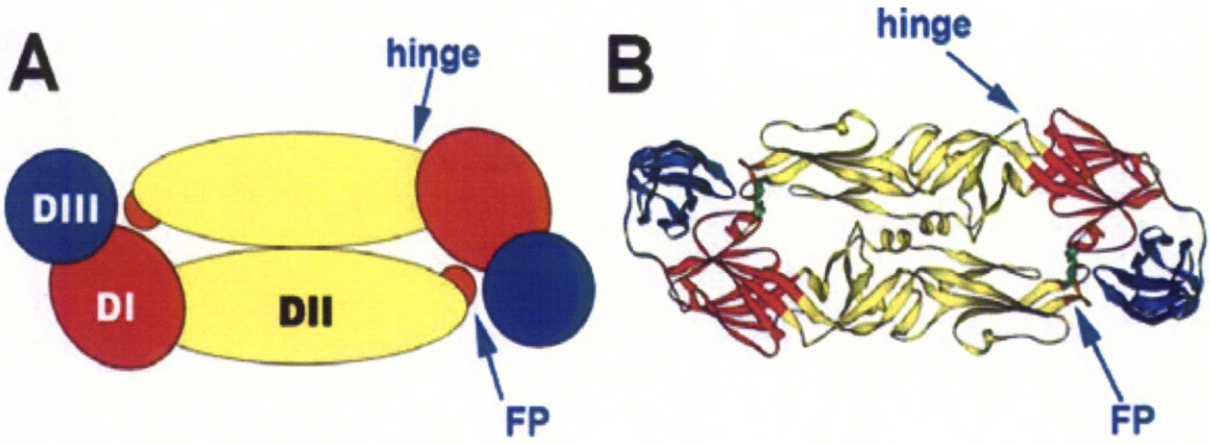


Figure 5-1. Structural organization of the flavivirus envelope protein. (A) Schematic top view of the organization of the E protein dimer as present at the surface of mature virions, color-coded according to the three domains (DI, DII, and DIII). The fusion peptide (FP) is indicated in orange. (B) Crystal structure (top view) of E ectodomain dimer. Taken from (Ren *et al.* 2007).

Neutralizing antibodies against domain III can inhibit a postattachment step of viral entry, and a soluble form of domain III can act as a potent dominant-negative inhibitor of this conformational change (Liao and Kielian, 2005).

The differences in the conformations of residues in domain III may help us to understand the variations in antigenic and neutralizing properties of JEV strains. Most of the phenotypically variant properties, like serotypic differences, variants of vaccine strains, and neutralisation escape variants, map with domain III (Cecilia and Gould, 1991; Gritsun et al., 1995).

The 3D structure of the E glycoprotein of the Muar strain was predicted and the difference and the critical changes between the Muar and other JEV strains were identified so this may help us to understand the variations in antigenic and neutralizing properties of different JEV strains.

## 5.2 MATERIALS & METHODS

### 5.2.1 Determining the Signature Amino Acids of the Muar Strain Glycoprotein

The signature amino acids of the E glycoprotein of the Muar strain were determined through alignment of 292 other JEV strains belonging to different genotypes.

### 5.2.1. Prediction of Three-dimensional Structure of the E Glycoprotein of the Muar Strain

The Muar-specific amino acids of the E glycoprotein, which distinguish genotype V from other genotypes, were mapped onto the predicted 3D structure of the Muar E protein, as modelled based on predicted similarities to the homologus (77% identity) glycoprotein gene in WNV (PDB entry 2i69) using SWISS-MODEL via the ExPASy Ib server (Schwede et al., 2003) as the X ray structure of the E protein of JEV is not available. The predicted Muar E structure was viewed and manipulated using PyMol (http://pymol.sourcecforge.net/).

Critical changes in the E glycoprotein which are thought to be involved in receptor binding as described by (Ni and Barrett, 1998; Solomon et al., 2003b) were identified in genotype V, the Muar strain.

## 5.3 RESULTS

### 5.3.1 Structural Differences in E Glycoprotein among the Muar and Other JEV Strains

Examination of the E protein of the Muar strain showed it was similar to other JEV strains. It consists of 500 amino acid residues. All the twelve cysteines that form disulfide bonds are conserved between the Muar strain and other JEV strains.

Alignment of the E glycoprotein of 292 JEV strain sequences revealed that there are twenty two Muar signature amino acids which are identified in Figure 5-2.

The range of intergenotypic amino acid sequence divergence of E glycoprotein between genotype V strain, Muar, and the other JEV genotypes was 8.5 to 9.7%.

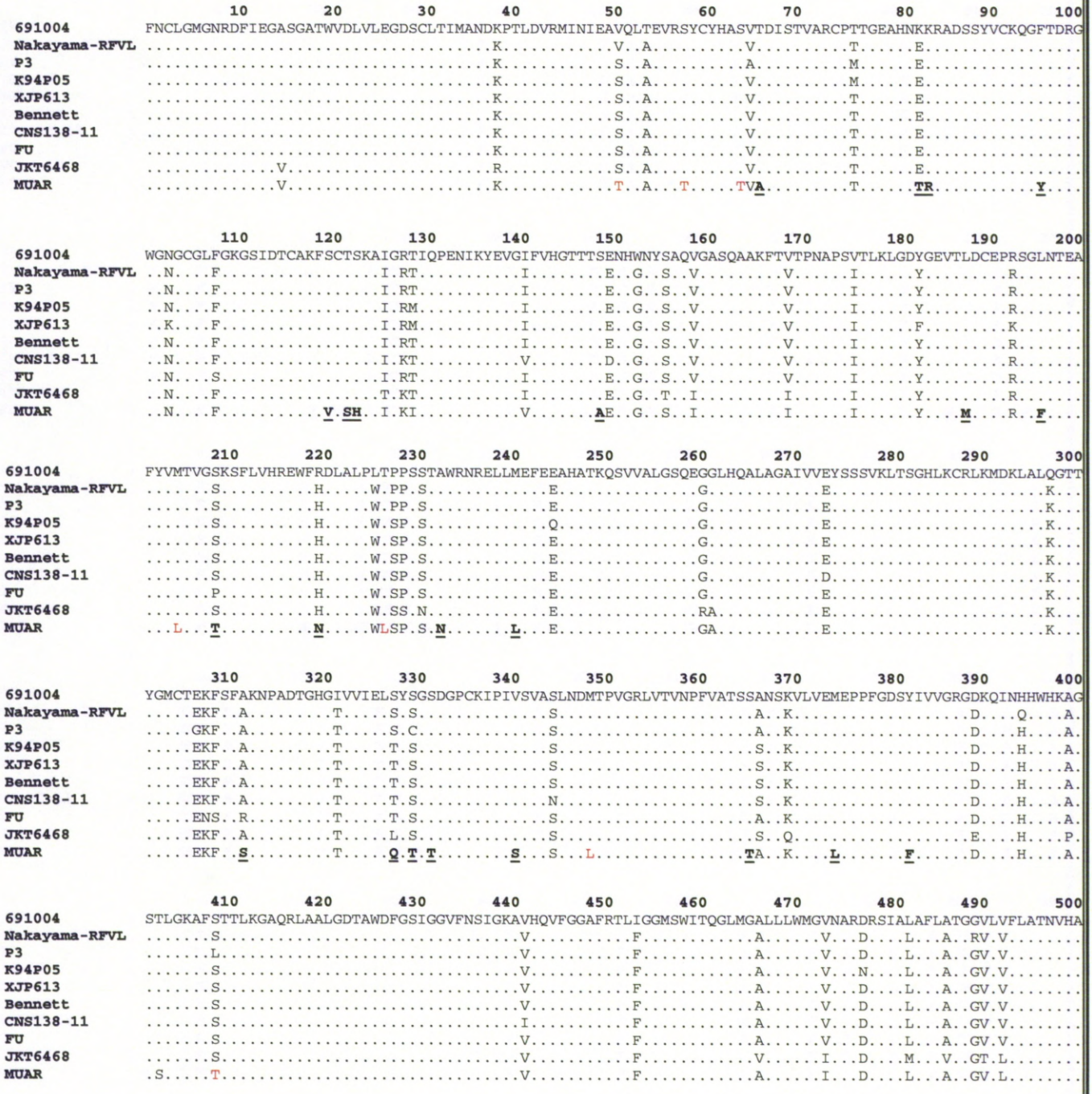


Figure 5-2. Alignment of the E protein amino acid sequences of JEV GI - V. Dots indicate consensus. Underlined amino acids represent the Muar signature amino acids.

*** Red colored amino acid residues are not signature amino acids for Muar strain as they are similar to other JEV strains which are not shown here.**

### 5.3.2 Variation within the E protein Tertiary Structure of the Muar strain of JEV

The predicted three-dimensional (3D) structure of the E protein of the Muar strain was modeled onto the crystal structure of WNV (Nybakken et al., 2006) as shown in Figure 5-3.

A comprehensive alignment of E genes from 292 JEV strains identified twenty two signature amino acid residues that distinguished genotype V from other genotypes; two signature amino acids for the Muar strain in domain I as shown in Figure 5-4, twelve in domain II as shown in Figure 5-5 and eight in domain III as shown in Figure 5-6 but the critical change is in E327 mapped to the exposed lateral surface of domain III in a region thought to be involved in receptor binding as described previously (Ni and Barrett, 1998 ; Solomon *et al.*, 2003b).

Interestingly, some of the Muar signature amino acids are similar to MVEV when MVEV is included in the comprehensive alignment of E genes of JEV strains including the Muar strain. Those amino acides that are unique to Muar in comparison with other JEV stains, but some of them are conserved with MVEV domains II and III as shown in Figures 5-8 and 5-9 respectively.

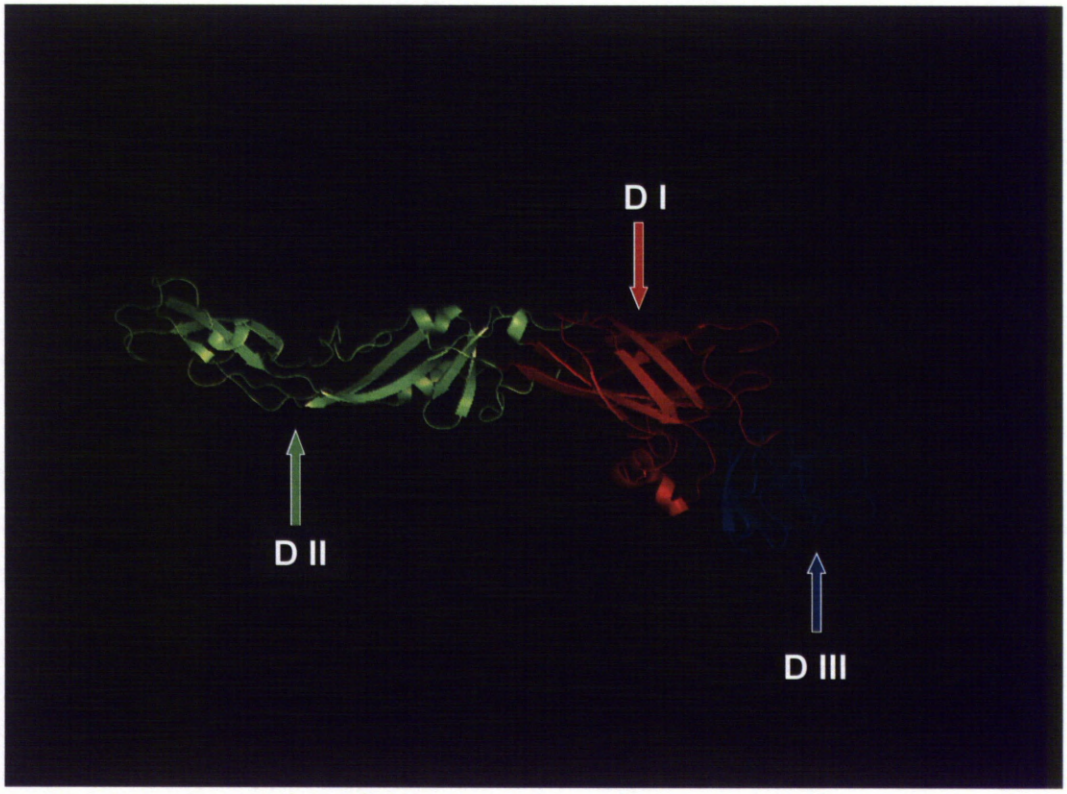


Figure 5-3. Predicted three-dimensional model of the E protein of JEV GV strain Muar, derived from the crystal structure of WNV, Individual E monomers are colored red (domain I), green (domain II), and blue (domain III).

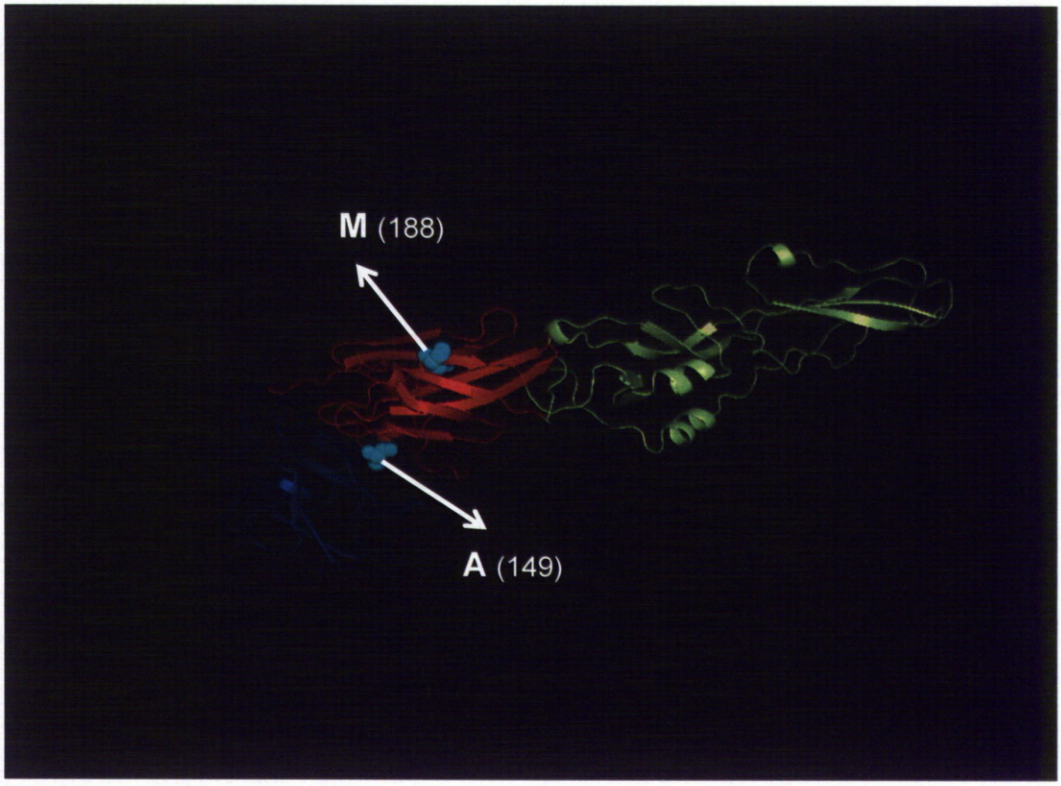


Figure 5-4. JEV E protein domain I. Predicted three-dimensional model of the E protein of JEV GV strain Muar, derived from the crystal structure of WNV, showing two Muar signature amino acids compared to other JEV strains.

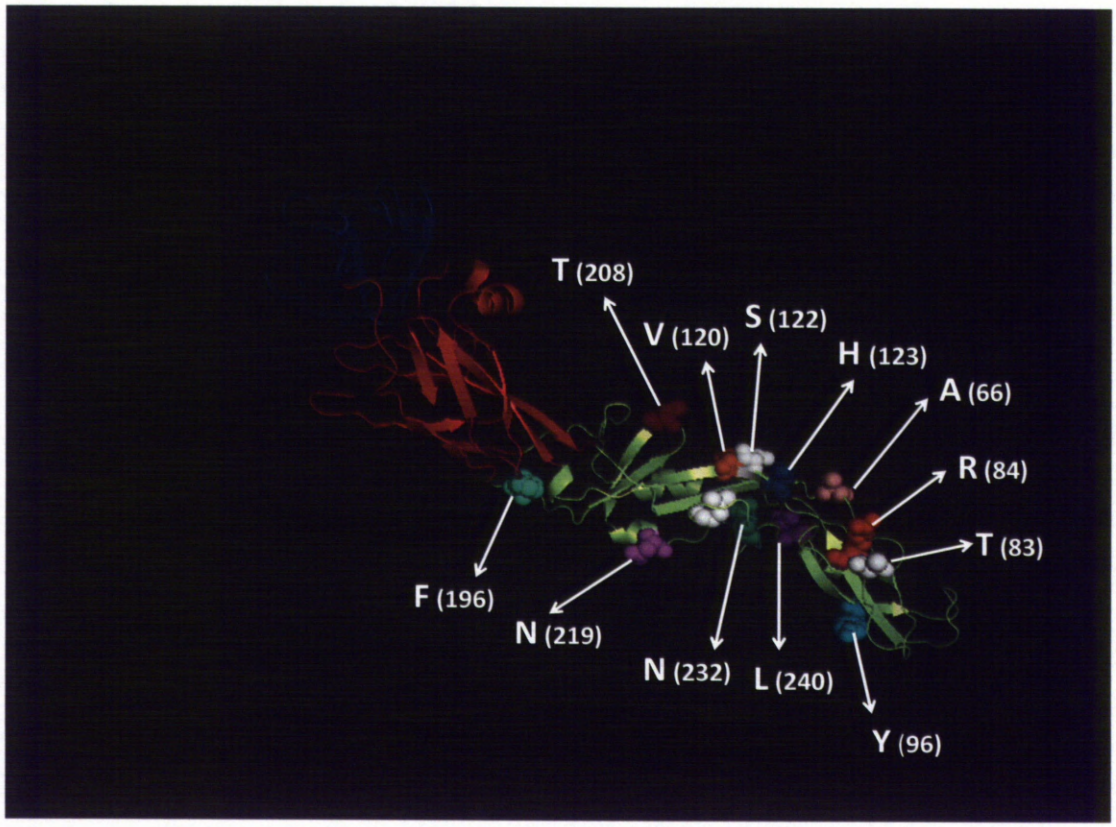


Figure 5-5. JEV E protein domain II. Predicted three-dimensional model of the E protein of JEV GV strain Muar, derived from the crystal structure of WNV, showing twelve Muar signature amino acids compared to other JEV strains.

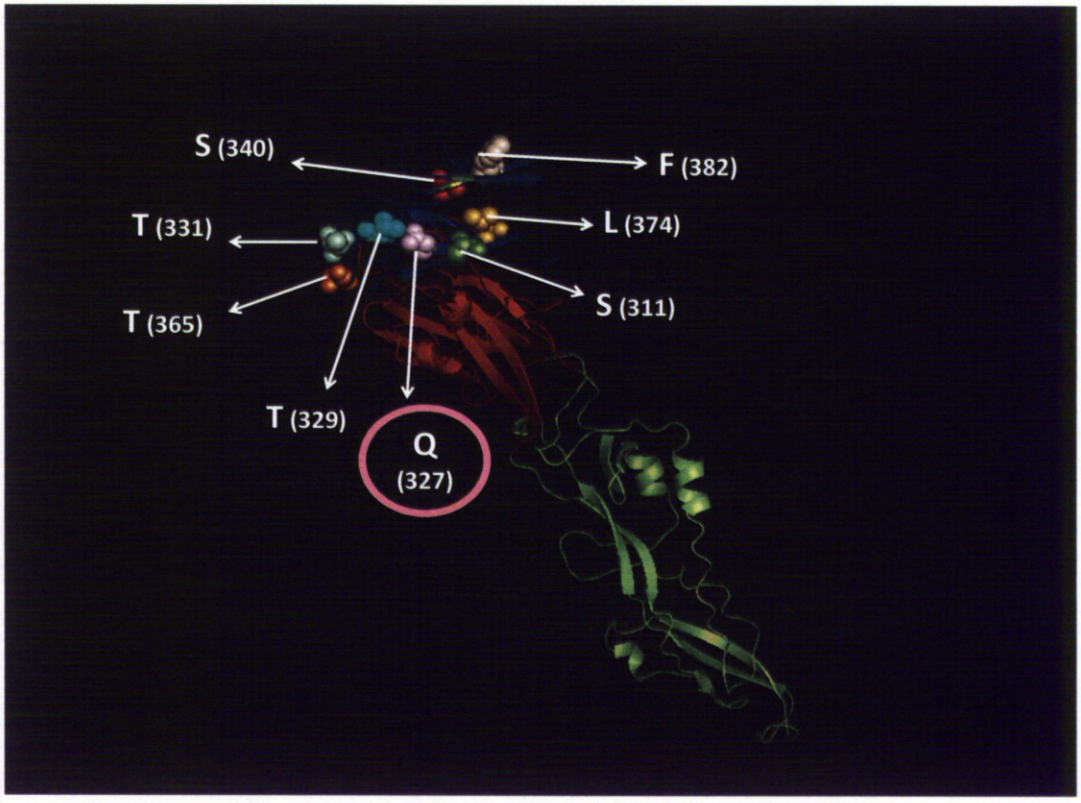


Figure 5-6. JEV E protein domain III. Predicted three-dimensional model of the E protein of JEV GV strain Muar, derived from the crystal structure of WNV, showing eight Muar signature amino acids compared to other JEV strains; the critical change at residue 327; circled, (in the putative receptor binding region of domain III) from the bigger size glutamine amino acid in genotype V strains to the aliphatic amino acid leucine in genotype IV strains then to serine and threonine amino acids found in newer genotypes; I, II and III.

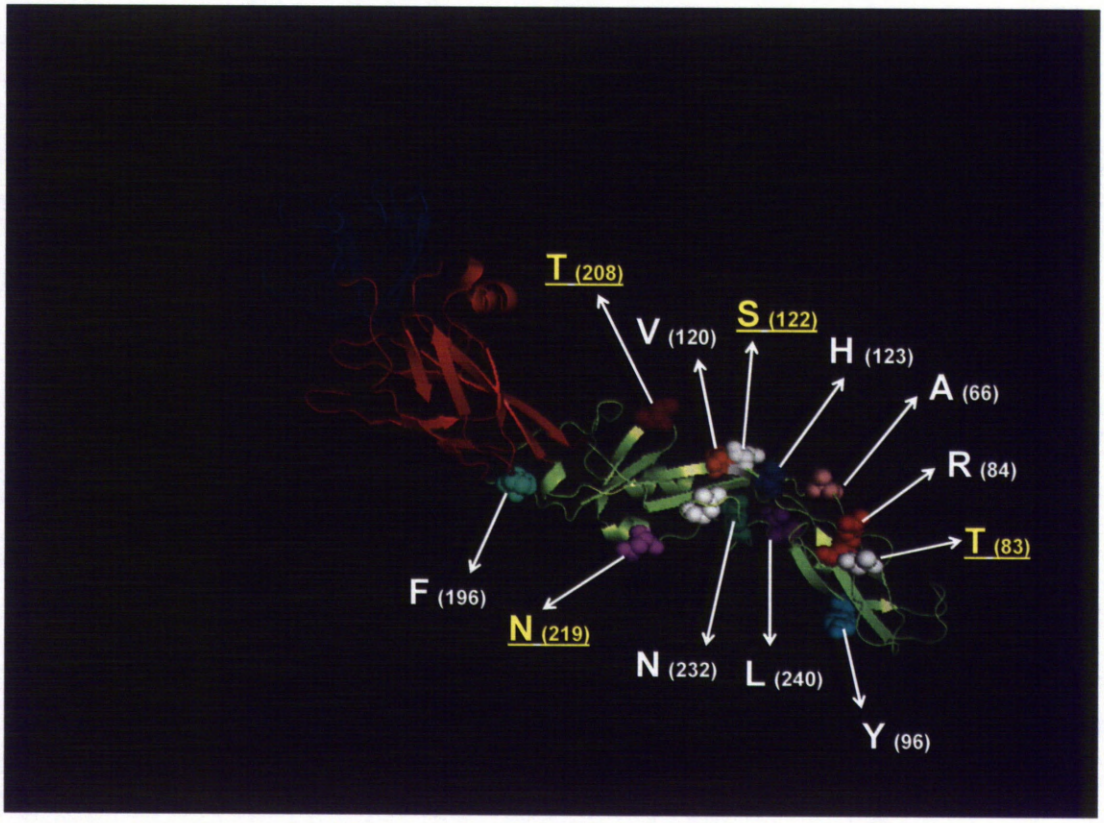


Figure 5-7. JEV E protein domain II. Predicted three-dimensional model of the E protein of JEV GV strain Muar, derived from the crystal structure of WNV, showing twelve Muar signature amino acids compared to other JEV strains, however, four of these amino acids are similar to MVEV (underlined).

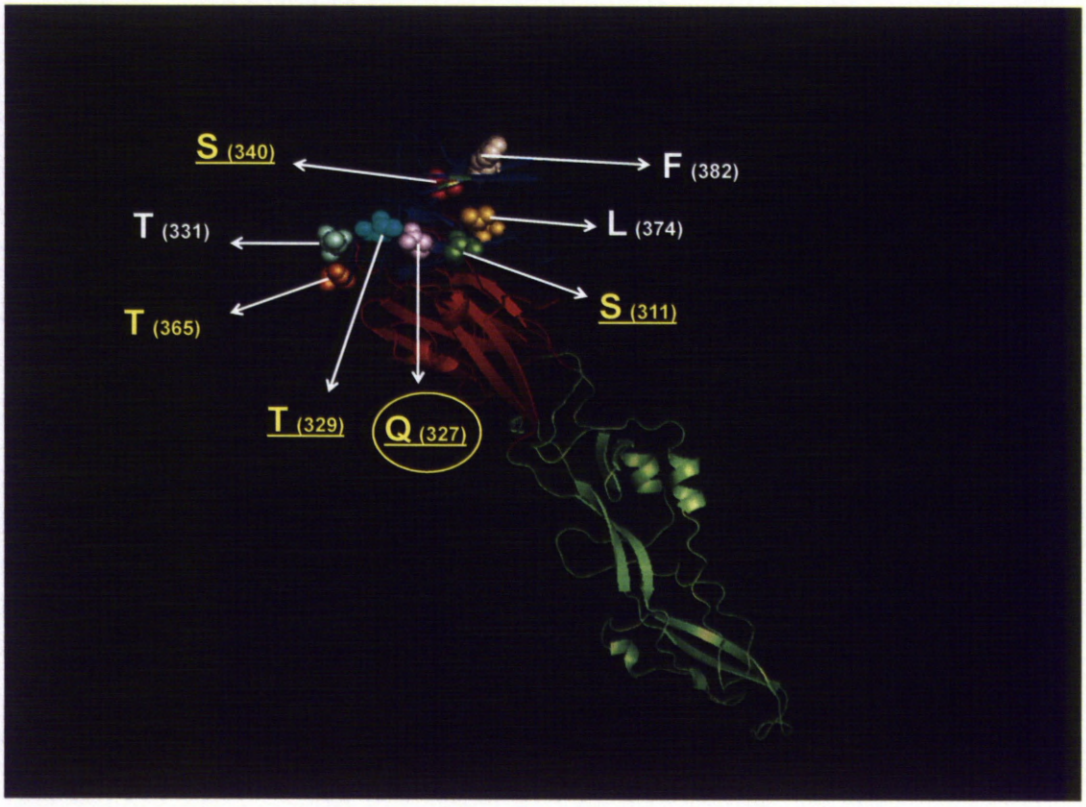


Figure 5-8. JEV E protein domain III. Predicted three-dimensional model of the E protein of JEV GV strain Muar, derived from the crystal structure of WNV, showing eight Muar signature amino acids compared to other JEV strains, however, five of these amino acids are similar to MVEV (underlined).

## 5.4 DISCUSSION

The E protein of the flaviviruses is the major antigen for theses viruses and is also believed to be associated with virus binding and entry to host cells. Thus, the majority of serological and genetic analyses of JEV have focused on the E protein.

Analysis of the Muar E glycoprotein revealed that it is similar to the E glycoprotein of other JEV strains. The E glycoprotein of JEV consists of 500 amino acid residues which form three domains (Rey *et al.*, 1995). Domain I, which is referred to as the central domain, consists of 128 residues from 1-51, 137-196, and 293-311. There are two disulphide bonds in this domain (Cys 3-Cys 30 and Cys 193-Cys 297). This domain contains the glycosylation site in addition to the epitopes with serological or biological activities. The second domain, domain II, consists of 171 residues (52-136 and 197-292) and three disulphide bonds (Cys 60-Cys 121, Cys 74-Cys 105 and Cys 92-Cys 116). This domain is called the dimerisation domain. Domain II has a hydrophilic region and contains epitopes involved in neutralisation and hemagglutination. The region 98-111 is a highly conserved region among flaviviruses, has a $\beta$-hairpin motif, and has been suggested as the one involved in the fusion activities (Roehrig et al., 1990). Domain III is a contiguous stretch of 100 residues (310-411) with only one disulphide bridge (Cys 314-Cys 346).

Site-directed mutagenesis studies of domain III proved that it is an important domain not only in JEV but also in other viruses such as MVEV, TBEV, and Louping ill virus (Cecilia and Gould, 1991; Holzmann et al., 1990; Jiang et al., 1993; Lobigs et al., 1990). Domain III has also been suggested to be involved in the receptor binding activities. The 399-RGD-401 sequence motif, present in domain III, is unique to the mosquito flaviviruses and was proposed to form part of the receptor binding site (Lobigs *et al.*, 1990).

The observed difference in the predicted 3D structure of E glycoprotein of JEV strains, particularly in domain III, could help in understanding the antigenic and serological properties of these strains (Cecilia and Gould, 1991; Gritsun et al., 1995). The differences in the conformations of residues in domain III may help us to understand the variations in antigenic and neutralizing properties of JEV strains. Most of the phenotypically variant properties, like serotypic differences, variants of vaccine strains, and neutralisation escape variants, map with domain III (Cecilia and Gould, 1991; Gritsun et al., 1995).

The choice of E protein for analysis also provides the best chance of finding variability related to serotypic groups of the virus. The bulk of the antibody response in JEV infections is directed against the E protein (Monath and Heinz, 1996). Variability in this region should therefore be directly correlated to changes in viral surface epitopes and therefore the presence of multiple serotypes of the virus.

Domain III of the E protein of JEV has been shown to contain important sites for antibody-mediated virus neutralization and motifs associated with virulence (Rey et al., 1995; Wu et al., 2003). Change in E327 in domain III of JEV has been considered as critical change as it is located in a region thought to be involved in receptor binding as showed in genotype IV strain of JEV (Ni and Barrett, 1998 ; Solomon et al., 2003b). Therfore, I could explain why the Muar strain is unique serollogicaly, becuse it has eight signature amino acids differences at domain III with a critical change at residue E327 (in the putative receptor binding region of domain III).

Why the Muar strain, the most ancient lineage of JEV has not spread is uncertain. JEV uses a wide range of mosquito species and vertebrate hosts across Asia, and geographical differences in vector and host availability may explain why genotype V, the Muar strain, has never spread. I examined the virus' complete amino acid sequence to look for molecular determinants that might relate to differences in host preference. I found many changes in the E protein, and a comprehensive alignment of E proteins identified twenty two signature amino acids. Depending on previous studies which showed that a small number of changes in E protein sequence could affect mosquito oral infectivity in arthropod borne viruses (Chen and Beaty, 1982; Ni et al., 1994); Tsetsarkin et al, 2007), I could explain why genotype V, is confined to Malaysia and has not spread elsewhere.

It has been found that few changes in the E glycoprotein amino acids could be responsible for the virus spread into new geographical areas, as for example, the sequence of the Australian FU strain (GII) predicts five amino acid changes within the E protein, four of which lie within predicted antigenic domains. Three of the mutations lie in close proximity (residues E307, E308, E311) and are in the region of an exposed loop that lies on the outside face of the E protein domain III (Rey et al., 1995b).

Interestingly, for the arthropod-borne alphavirus, Chikungunya virus (CHIKV), single amino acid changes in the E protein have been shown to be associated with change in vector competence. Although the typical vector for CHIKV is *Aedes aegypti,* a single mutation in the E protein gene was directly responsiple for CHIKV adaptation to *Ae. albopictus* mosquitoes (Tsetsarkin et al, 2007).

It has been suggested that there may be a minimum amino acid difference threshold that must be crossed to loose cross-protection; i.e. the occurrence of a different serotype. This heterogeneity threshold appears to be about 20% (Tsarev et al., 2000). The amino acid differences between JEV strains including the Muar strain are consistent with one serotype hypothesis. The maximum observed differences was less than 10%. Overall, the genetic analysis presented here suggests that only one serotype of JEV exists indicating that JEV vaccines are effective against different JEV genotypes.

Interestingly, I showed that some of the Muar signature amino acids in domains II and III, which are different from other JEV strains, are similar to MVEV, the closest flavivirus to JEV, when MVEV is included in the alignment of E genes of JEV including the Muar strain. This indicates that the Muar strain, the most ancient JEV strain, could evolve from MVEV.

Analysis of the E glycoprotein of the Muar strain of JEV may help to provide pointers as to which critical amino acids might explain the serological differences between genotype V and other more recently evolved genotypes; it may also help explain why genotype V appears not to have been as successful as other, more recently evolved genotypes, in terms of circulation and spread. Experimental studies with an infectious JEV clone, looking at the effect of particular mutations on mosquito infectivity and transmissibility would help explore this further.

# CHAPTER 6: INVESTIGATING NEUTRALIZATION EFFECTIVENESS OF INTRA VENEOUS IMMUNOGLOBULINS FROM AN ENDEMIC PART OF ASIA ON FIVE DIFFERENT GENOTYPES OF JEV

## 6.1 INTRODUCTION

JE is the most common cause of viral encephalitis in Asia; 30,000–50,000 human cases are reported annually. Clinical disease presentations range from a flulike fever to a severe and deadly meningoencephalitis with a parkinsonian syndrome. The mortality rate is high (range, 25%–30%), and neuropsychiatric sequelae occur in 50% of patients (Solomon and Winter, 2004).

Following infection with JEV, the ratio of symptomatic to asymptomatic disease is estimated to be 1:50-1:300 (Libraty et al., 2002a). JEV targets the CNS causing encephalitis as well as meningitis (Hosokawa et al., 2007).

Therapy for JE is supportive and no clearly effective specific antiviral agents exist (Mishra and Basu, 2008). Complications of infection such as seizures and raised intracranial pressure need to be identified and treated. Careful nursing care and physiotherapy are needed to reduce the risk of bedsores, malnutrition and contractures. Aspiration pneumonia is common in patients with a reduced gag reflex.

Experimental laboratory studies showed that some compounds have shown good efficacy against JEV such as isoquinolones, anti-JEV MAbs, nucleoside analogs, the lymphocyte modulator concanavalin A, nitric oxide and ribavirin (Harinasuta et al., 1984; Kimura-Kuroda and Yasui, 1988a).

IFN-α has been the most promising antiviral candidate for most of viral infections including JEV (Burke & Morill, 1987). However, a double-blind placebo-controlled trial in Vietnamese children with JE showed that although IFN-α may have delayed the time of death, it made no effect on the overall outcome (Solomon, 2003a).

IVIGs have been used recently to treat flavivirus encephalitis. In a case report, IVIG was shown to be of benefit when administered with symptomatic treatment for JE. The patient improved rapidly with complete recovery (Caramello et al., 2006).

A previously healthy 49-year-old Italian man was admitted to the hospital after a 3-week trip to rural Vietnam. At admission, he was stuporose, somnolent, disoriented, and febrile (39.5 °C) and had mild meningismus, photophobia, and conjunctival hyperemia. The following signs were observed: fluctuating responsiveness, mask-like facies, orofacial dyskinesias, slurred speech and motor aphasia, ataxia, intentional tremors, and an inability to perform manual procedures. Analysis of a CSF specimen revealed a white blood cell (WBC) count of 400 cells/mL. Serologic testing was performed by immunofluorescence, and the results were positive for JEV, with seroconversion in subsequent samples of serum and CSF; peak titers are reported. MRI (magnetic resonance imaging) revealed asymmetrical, T1-lighted hypointense and T2-lighted hyperintense lesions at the right lentiform nucleus and bilateral thalamus, without contrast enhancement. Electroencephalography revealed diffuse theta-delta activity and generalized slow waves. Supportive therapy with saline and NSAIDs was administered, but a worsening of confusion and agitation were observed. On day 6 of hospitalization, a 5-day course of IVIG were initiated. The patient started to improve after receiving the first perfusion, with progressive recovery. On day 23, the patient was oriented but still slightly slurred in mentation, motor activity, and speech. However, a month later, only a slight deficit in recent memory was observed. This was the first case of the use of IVIG to treat JE infection (Caramello *et al.,* 2006).

Because of the interest in IVIG as potential therapy for JE, I decided to investigate neutralising antibody titres in commercially available IVIG from India where JE is endemic. This is especially timely because IVIG is now being used on a presumptive basis in some cases of JE. In India genotype III of JEV has been circulating since at least the 1950s; there are outbreaks every year, and very large outbreaks every few years. I was particulary interested in whether the available IVIG had neutralisation activity against the Muar strain.

## 6.2 MATERIALS & METHODS

### 6.2.1 Neutralization Assays

The neutralization effectiveness of three different human IVIGs (Table 6-1), marketed in India, from pooled donors who are JEV antibody-positive were investigated against a strain of each of five JEV genotypes. JEV strains were chosen based on the lab availability; 2372 from genotype I, CNS138-11 from genotype II, P3 from genotype III, JKT6468 from genotype IV, and Muar from genotype V.

Table 6-1. Source of the intravenous immunoglobulins used in this study

| IVIG | Patented in: | Manfactured By: | Batch No. |
|---|---|---|---|
| A) | India | BHARAT SERUMS AND VACCINES LIMITED | A3306004 |
| (B) | India | CLARIS LIFESCIENCES LIMITED | 200609032 |
| (C) | India | BHARAT SERUMS AND VACCINES LIMITED | A3307017 |

### *6.2.1.1 Passaging Virus*

JEV strains were passaged four times in Vero cells in our containment level 3 (CL3) lab before subsequent use. Supernatant from infected cells was added to a confluent monolayer of Vero cells, left for 30 minutes at room temperature before adding maintenance media containing 5% FBS DMEM supplemented with glutamine, penicillin/streptomycin. Supernatant was collected two days later and frozen at -80 $^{o}$C.

### *6.2.1.2 Plaque Titration*

Infectivity of JEV strains were measured by plaque titration using 6-well tissue culture plates containing confluent Vero cell monolayers. The virus was added to the cells in ten-fold dilutions and left at room temperature for 30 minutes, rocking the plates every five minutes. After this time, 4 ml of 2% agarose/MEM overlay was added to the cells and the plates were placed at 37$^{o}$C in a $CO_2$ incubator, with 5% $CO_2$ level, for 5-6 days before fixing with formalin and staining with crystal violet. Figure 6-1 shows the ten-fold virus dilutions.

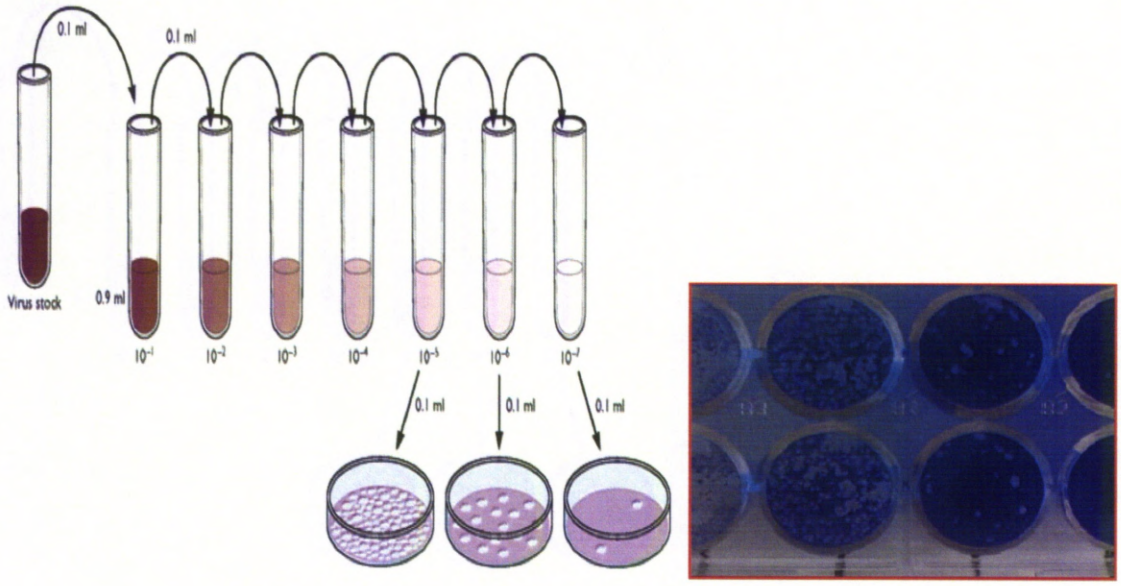


Figure 6-1. The ten-fold virus dilutions used for plaque titration.

### *6.2.1.3 Plaque Reduction Neutralization Test (PRNT)*

Fifty % plaque reduction neutralization titers ($PRNT_{50}$) were determined for each IVIG. The $PRNT_{50}$ test was performed essentially as originally described by De Madrid and Porterfield (De Madrid and Porterfield, 1974).

Briefly, the IVIGs were diluted (1/5) then followed by twofold serial dilutions. Subsequently, each dilution was mixed with an equal volume of virus preparation "~ 30 plaque forming unit (pfu) per well" and kept for half an hour at 37 °C in a $CO_2$ incubator. Aliquots of 100µl of the mixtures were inoculated in duplicate in 6 well plates of a Vero cell monolayer and kept at 37 °C for half an hour with four occasional agitations. Agarose overlay media were then added, and the cells were kept at 37 °C in a $CO_2$ incubator for 5-6 days before fixing with formalin and staining with crystal violet.

The highest IVIG dilution with plaque reductions of 50% was defined as the titration end point. IVIGs that showed 50% plaque reduction at 1:10 dilution or more were considered to be positive (Monath et al., 2000).

## 6.3 RESULTS

### *6.3.1Plaque Reduction Neutralization Tests ($PRNT_{50}$)*

The ability of IVIG to protect against strains of JEV representing the five major genotypes was assessed. Neutralization assays showed IVIGs appear cross-reactive across the five JEV genotypes, with effective but lower titres for the older genotypes IV and V.

For IVIG (A), the $PRNT_{50}$ ranged between 120 in genotypes IV and V to 640 in GIII as shown in Figure 6-2.

For IVIG (B), the $PRNT_{50}$ ranged between 160 in genotypes IV and V to 640 in G III as shown in Figure 6-3.

For IVIG (C), the $PRNT_{50}$ ranged between 80 in GV to 320 in genotypes I and III as shown in Figure 6-4.

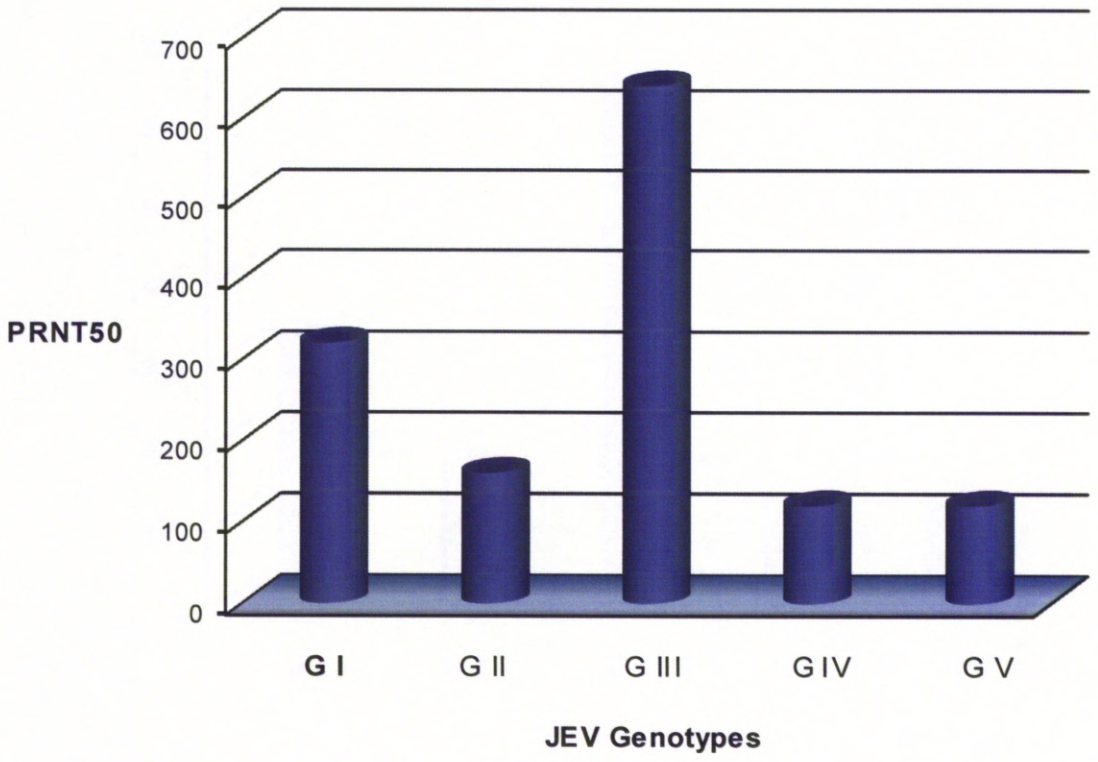


Figure 6-2. $PRNT_{50}$ for a representative strain of each JEV five genotypes against IVIG (A).

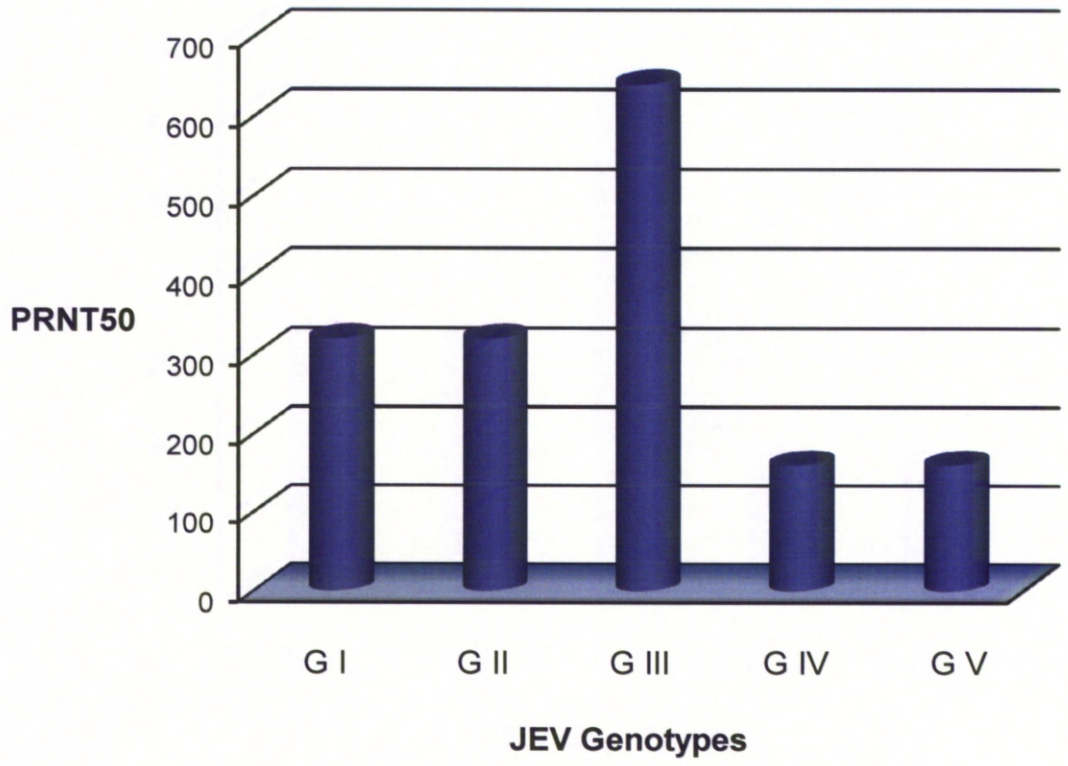


Figure 6-3. $PRNT_{50}$ for a representative strain of each JEV five genotypes against IVIG (B).

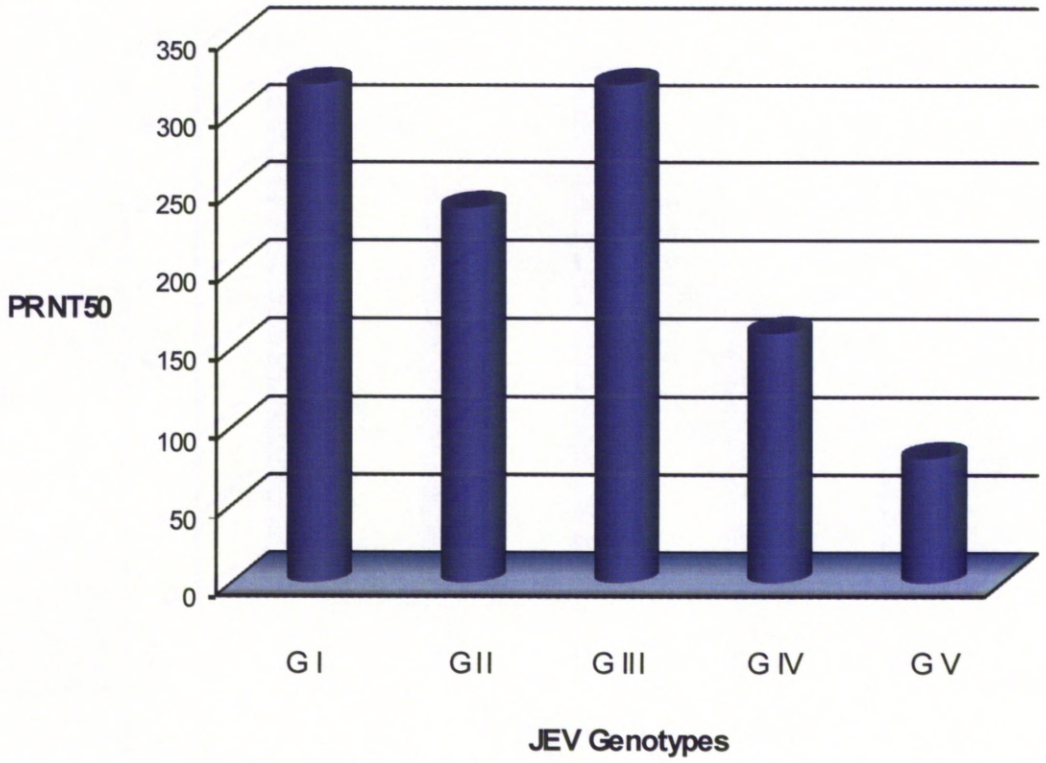


Figure 6-4. $PRNT_{50}$ for a representative strain of each JEV five genotypes against IVIG (C).

## 6.4 DISCUSSION

There is no known effective, specific treatment for JE. However, a case report of JEV has been treated with IVIG and clinical improvement was rapid and constant during the course of treatment and resulted in almost complete recovery (Caramello *et al.*, 2006).

For patients with infectious diseases, IVIG is administered with two aims. The first aim to increase viral clearance due to antibody dependent neutralization, as is used for example in immunodeficient patients (e.g., in cytomegalovirus-infected bone marrow transplant recipients or parvovirus-infected subjects with HIV infection) or in patients with specific infections (e.g., SLEV or WNV infections) (Agrawal and Petersen, 2003; Bayry et al., 2003). The second aim for high-dose IVIG, is to benefit patients with certain acute inflammatory diseases that have both viral and autoimmune causes (i.e., acute disseminated encephalomyelitis) (Nishikawa et al., 1999). However, in the latter case, the mechanism of action is not completely clear. IVIG has been found to inhibit the function of different arms of the immune system (i.e., maturation and function of dendritic cells, proliferation of T lymphocytes, and production of proinflammatory cytokines).

Passive transfer of specific antibodies or Igs has been shown to abort or modify a number of *Flavivirus* infections, including infection with JEV (Kimura-Kuroda & Yasui, 1988) or MVEV (Broom et al., 2000), SLEV (Mathews and Roehrig, 1984), TBEV (Phillpotts et al., 1987) and 17D YF (Schlesinger *et al.*, 1985). It appears, therefore, that the only currently available treatment for viral infection that could provide a state of immediate immunity is passive transfer of specific antibodies (Casadevall and Scharff, 1994; Rosas et al., 2001).

Antibodies play a major role in protection and recovery of infected mice from WNV infection and IVIG can be used as first-line therapy; full protection was (Ben-Nathan et al., 2003) achieved when the infected mice were treated with pooled human plasma (PP) or IVIG obtained from healthy Israeli blood donors that contained WNV-specific antibodies. Recovery of the lethally infected mice was dependent on the dose and time of IVIG administration (Ben-Nathan *et al.*, 2003).

In this chapter, the neutralization effectiveness of three different IVIGs on representatives from the five JEV genotypes was determined. These are currently being used as a supportive treatment for JEV infection in India as until now no clearly effective specific antiviral agents exist (Mishra and Basu, 2008). In a cell culture model, IVIGs reacted significantly with higher titers for genotypes III but appear cross-reactive against the other four genotypes as well. Given that so far all strains of JEV isolated in India are genotypes III strains, this is not unexpected. The results of the study are consistent with previous studies (Ali and Igarashi, 1997; Wills et al., 1993), and suggest that the virus used in neutralization assays affect the antibody titre obtained. $PRNT_{50}$ titers vary depending on the virus strain used in the neutralization tests, namely higher $PRNT_{50}$ titers were obtained when the challange virus was homologus to the vaccine strain compared to use of a heterologous virus (Ferguson et al., 2008).

Cross-neutralization experiments with sera raised against JEV strains of different genotypes and studies using MAbs have demonstrated antigenic differences both within and between genotypes of JEV strains (Ali and Igarashi, 1997; Wills et al., 1993). However, epidemiological evidence indicating that secondary CNS infections with JEV are not reported and the limited nucleotide and amino acid divergence of JEV strains suggested to some authors that all JEV strains including Muar comprise a single "serotype" (Markoff, 2000; Tsarev et al., 2000). Hence, exposure to virus of one genotype might be expected to protect against all JEV strains.

The $PRNT_{50}$ titers for all IVIGs against the five JEV strains ranged between 80 and 640, and no single IVIG neutralized all strains better than the others. In general, the highest titers for all three IVIGs were observed against strains of genotype III (titers of 320 and 640), while $PRNT_{50}$ titers against strains of genotypes IV and V ranged between (80 and 160). This observation is consistent with previous reports describing lower levels of neutralization against heterologous strains of JEV. It has been suggested that neutralizing antibody titers of $\geq 10$ are probably protective (Monath, 2002). The protection afforded by antibodies induced by experimental JEV vaccines in mice against JEV strains representing the four major genotypes provided protection against the virus challenge in the mouse model at $\geq 20$ (Beasley et al., 2004).

Our results indicate that, although neutralizing antibody titers were lower against genotypes IV and V viruses, IVIGs are capable of neutralizing strains of genotypes IV and V at protective levels.

To our knowledge, there is only one reported case of IVIG to treat JE. Additional investigations and clinical trials are warranted before any standardised recommendations should be made, but IVIG may prove to be useful when administered with symptomatic treatment for this deadly disease.

Investigation into new antiviral drugs against JE is proving to be one of several important areas of research stimulated by the growing threat of this spreading virus.

Because of the unavailability of specific treatment for JE, vaccination remains the most promising tool to reduce this deadly infection (Tsai, 2000).

# CHAPTER 7: GENERAL DISCUSSION

Great interest has been directed towards emergence and evolution of infectious diseases especially since the start of the acquired immune deficiency syndrome (AIDS) epidemic in early 1980s. It is clearly extremely important to know what factors allowed new infections like HIV to appear, or older ones to reappear, and then to track their spread through populations (Zuckerkandl, 1987).

One of the most basic questions to ask with any infectious disease is where it comes from. Although arthropod-borne encephalitis viruses including JEV represent a significant public health problem throughout most of the world, their origins and evolution remain unclear (Solomon and Cardosa, 2000).

Tracing the spread of infectious diseases was traditionally approached by serology, that is by studying the movement of pathogens strains which differed in immunological response. serological tests divided *Flavivirus* genus of the family *Flaviviridae* into eight serocomplexes, including TBE serocomplex, DEN serocomplex, and JE serocomplex. Several mosquito-borne neurotropic viruses of the JE serocomplex, cause encephalitis outbreaks in humans across the world including WNV which found in Africa and north into Europe, and recently in North America, SLEV which circulate in the United States, MVEV and KUNV which both are found in Australia and Rocio virus in Brazil. In addition, JEV, the most important cause of epidemic encephalitis worldwide in terms of numbers; causing 30,000 to 50,000 cases with 10,000 to 15,000 deaths every year (Tsai 2000).

JEV is endemic to Southeast Asia and surrounding Pacific Islands. However, its recent emergence in northern Australia (Mackenzie, Poidinger et al. 1997; Hanna, Ritchie et al. 1999) has caused great public health concern and global alert as this virus could expand its geographical range, spread to naive areas and become a worldwide public health threat.

Although JEV is confined to Asia and Australia at the moment, the possibility of it spreading anywhere else all over the world is high. The reasons for JEV spread may include changing agricultural practices, such as increasing irrigation, and animal husbandry (Tsai, 1997). In addition, migrating birds are thought to be important in the virus' dispersal to new geographic areas (Innes, 1995).

JEV continues to spread with recent outbreaks in India, Nepal, and Australia. The most recent large outbreak was reported in northern India in 2005 causing 5000 cases, with 1300 deaths (Solomon 2006). It is not known what went wrong in 2005 but it could be because of the vaccine unavailability, a late government response, and severe lack of medical support including medical staff, medicines and even beds.

The occurrence of JE- cases and JE-related deaths is increasing in recent years, suggesting that JE may be spreading to naive areas. This could be due to the ecological changes and the extensive traveling of people looking for employment and for social reasons. Despite several outbreaks, the knowledge gained from these epidemics is negligible. Therefore, collection of epidemiological information from JE epidemics from different places is inevitable to frame JE-control strategies (Kabilan *et al.*, 2004). Therefore, vaccination against JE ideally should be practiced in all areas of Asia where the virus is responsible for human disease.

JE emerged in Japan in 1870 and then spread across Asia to affect most of China and the Asian subcontinent, all of Southeast Asia, and the Pacific Rim, reaching northern Australian in 1998. It has become the most important cause of epidemic encephalitis worldwide and there is a clear need for a better understanding of the origins and spread of this virus.

In this study, I showed the possibility of JEV evolution from MVEV as NJ phylogenetic analysis in Figures 3-25 and 3-26 revealed the evolution of the oldest JEV lineage, Muar strain, from MVEV. Furthermore, Muar stain of JEV shared conserved amino acids residues in the E glycoprotein with MVEV as elucidated in Figures 5-7 and 5-8.

Epidemiological studies have demonstrated four genotypes of JEV (I - IV). Sequencing of the complete genome of the Muar strain showed that the Muar isolate was the most genetically different of the other JEV strains, in agreement with reactivities results using anti-Muar MAbs (Hasegawa, 1982; Hasegawa et al., 1995) confirming that Muar is unique both genetically and serologically.

Hence, Domain III of the E protein of JEV has been shown to contain important sites for antibody-mediated virus neutralization and motifs associated with virulence (Rey et al., 1995b; Wu et al., 2003); the eight signature amino acids differences at domain III with a critical change at residue 327 (in the putative receptor binding region of domain III), may

explain some of the serological differences with the Muar strain. However, in this study I showed that all JEV strains including Muar are belonging to one serotype by determing the amino acid divergence percentage within the E protein, suggesting that the available JEV vaccines are effective against different JEV strains and exposure to virus of one genotype might be expected to protect against all JEV strains. However, because of the high evolution rate of JEV associated with the lack the proof-reading ability of viral RNA polymerases, we could expect the appearance of newer genotypes in the future which might escape the vaccine protection.

The critical amino acid change at E327 is from glutamine in genotype V strain to leucine in genotype IV strains then to serine and threonine amino acids found in newer genotypes I, II and III. This amino acid change could play a role in altering vector and/or host preference and could have contributed to the wider dispersal of the newer JEV genotypes compared with those of genotype V. Previously a small number of changes in E protein sequence have been shown to affect mosquito oral infectivity (Chen and Beaty, 1982; Ni et al., 1994).

There is no doubt that phylogenetic trees have become an important analytical tool and they have been used to study the origin and spread of viral infections. In this study, phylogenetic and evolutionary analyses revealed that the Muar strain represents a fifth genotype of JEV. Furthermore, the Muar strain represents the oldest JEV lineage when compared with other genomic sequences. I showed that JEV arose from its ancestral virus in the Indonesia-Malaysia region since mid 1500s and evolved into five genotypes.

The Indonesia-Malaysia region had the most divergent genotypes (IV and V) representing the oldest lineages and this region is geographically close to Australia, where MVEV, the closest relative to JEV, is found. Thus JEV originated in the Indonesia-Malaysia region from an ancestral virus common to JEV and MVEV. From this ancestral virus JEV genotypes IV and V diverged, followed by the more recent genotypes I, II and III. This is confirmed by the fact that the more recently divergent genotypes have spread to other areas, whereas the more divergent genotypes (IV and V) appear geographically confined to the Indonesia-Malaysia region (Solomon et al., 2003b).

Muar strain represents the only isolate of genotype V virus. However, we are not sure whether the Muar strain isolation was the results of a genotype V epidemic focus that quickly

died off, or a genotype V strain that circulated in Malaysia for a period of time prior to extinction, or the Muar strain was a single imported case from another region.

We are not sure why the Muar strain, the more ancient lineage of JEV, has not spread but the host availability may help to explain this. For the arthropod-borne alphavirus, CHIKV, single amino acid changes in the E protein have been shown to be associated with a change in vector competence. Although the typical vector for CHIKV is *Aedes aegypti*, one mutation in the E protein gene has been shown to be responsiple for CHIKV adaptation to *Ae. albopictus* mosquitoes (Tsetsarkin et al, 2007).

JEV is a serious encephalitic disease which is caused by RNA virus that has high evolutionary rate which could lead to appearance of new genotypes and the more JEV diversity the harder it is to control. Understanding the immune responses against JEV's prevention and recovery is very important to the development of new vaccines and specific treatment. Our present knowledge regarding JEV immune response is incomplete and some of the issues that remain to be addressed towards a fuller understanding of immune responses to JEV such as the effect of flavivirus cross-reactive immunity in the protection against and pathogenesis of JE, the role of CTLs in infection by JEV, protective immunity against heterologous virus strains, protective immunity to be elicited by vaccine, protective immunity elicited by NS1 and roles of nonspecific immune responses.

Because of the complex life cycle of JEV, involving mosquito vectors and multiple vertebrate hosts, we do not expect that this virus is going to disappear altogether. However, further research, studying the pathogenesis of this virus, will help to the development of specific antiviral therapy and effective, safe and cheaper vaccine which could be administered orally as a single dose.

JE is the most vaccine-preventable disease, therefore vaccination against it ideally should be practiced in all areas of Asia where the virus is responsible for human disease and travellers to endemic areas should be vaccinated too. Inactivated viral vaccines prepared in mouse brain or cell culture have been used effectively for the last several decades to immunize travellers and residents of endemic countries. However, cost and safety concerns including allergic and neurologic complications, led to development of live attenuated virus vaccine (SA14-14-2) and more recently, a purified inactivated, tissue culture-based Japanese encephalitis vaccine

(IXIARO®) has been licensed in the United states, Europe, Canada and Australia. In addition, IMOJEV®, a new vaccine was recently licensed in Australia and is under review in Thailand, which is a novel recombinant chimeric virus vaccine, developed using the yellow fever virus vaccine vector (YFV 17D), by replacing the cDNA encoding the envelope proteins of yellow fever virus with that of an attenuated Japanese encephalitis strain (SA14-14-2). IMOJEV was found to be safe, highly immunogenic and a single dose was sufficient to induce protective immunity, which was similar to that induced by three doses of a mouse brain derived inactivated JE vaccine. There is no doubt that these new vaccines, when appropriately administered, will greatly diminish the burden of disease (Bharati and Vrati, 2010).

Up to date, there is no licensed therapy available for JEV infection. Vaccination is considered to be the most effective control method. Thus, the WHO called for the development of novel, safe and more efficacious JE vaccines. Understanding JEV immunopathogenesis will not only help us to devolp more safe and cheap vaccine but also specific anti JEV therapy.

In summary, the complete genome sequence analysis of the Muar strain of JEV confirms that this strain represents a fifth genotype of JEV with approximately 20% nucleotide sequence divergence from other JEV strains. Evolutionary analysis suggested Muar represents the oldest JEV lineage and the four genotypes diverged from genotype V approximately at mid 1500s. I showed the possibility of evolution of JEV from MVEV. I looked at the pattern of the geographical distribution of JEV isolates using the E gene and I showed that JEV strains are completely mixed up. Furthermore, intensive E gene sequence analysis of the Muar strain of JEV via determing its signature amino acids when compared with other JEV strains revealed why the Muar strain of JEV is different serologically from other JEV strains as it is representing the fifth antigenic group and it also explained why the Muar strain is confined to the Indonesia-Malaysia region and has not been found elsewhere. In addition, I found that the E protein of all five JEV genotypes is subjected to strong negative selection pressure, so selection pressure is unlikely to be the reason for the genotypic shift of this virus. Interestingly, I showed the high homology of the terminal nucleotide sequence of the 5' and 3' NCRs of JE serocomplex, JEV, MVEV, WNV and SLEV, suggesting the important role played by these regions for viral proliferation. Prediction of the secondary structure of the 5' and 3' NCRs suggested that JEV genome ends could stick to each other. Finally, I showed *in vitro* that IVIGs are capable of neutralizing all five JEV genotypes at protective levels so they could be used for treatment of JEV but further *in vivo* studies are needed to know the exact

dose and time of IVIGs administration. Understanding the immune response to JEV will help us to develop more specific and effective treatment therefore; vaccination remains the only way to protect from this disease.

# APPENDIX

## APPENDIX A

***Nucleotide Sequence of 5` NCR of the Muar Strain of JEV***
***(From 1 to 95)***

5' AGAAGTTTATCTGTGTGAACTTCTTGACTTAGTATCGTTGAGAGGAA
TCGAGAGATTAGTGCAGTTTAAACAGTTTTTTAGAACGGAAGAAAACC
3'

***Nucleotide Sequence of C Gene of the Muar Strain of JEV***
***(From 96 to 476)***

5' ATGACTAAAAAACCAGGAGGGCCCGGTAGAAACCGGGCTATCAATA
TGCTGAAACGCGGTTTACCCCGCGTATCCCCACTTGTGGGGGTGAAGA
GGGTAATAATGAACTTGCTGGACGGCAGAGGGCCAATACGATTCGTTT
TGGCTCTCTTGGCGTTTTTCAAGTTCACAGCATTGGCCCCAACTAAGGC
ACTTATTAGCCGATGGAAAGCAGTAGAGAAGAGCGTCGCGATGAAAC
ACCTCACCAGCTTCAAAAAGGAACTGGGAACGCTCATCAACGCTGTGA
ATAAGAGGGGCAAAAAACAAAACAAAAGAGGAGGAAGTAATGGAACA
ATTATTTGGATGATAGGTTTGGCAGTCGTGTTCGCCACTGTGAGTGCA
3'

***Nucleotide Sequence of prM Gene of the Muar Strain of JEV***
***(From 477 to 977)***

5' GTCAAGCTGTCAAACTTTCAGGGCAAGGTGCTGATGACAATCAATAA
CACCGACGTGGCTGATGTGATCACCATTCCCACCTCGAAAGGGACCAA
TAGATGTTGGGTTCGGGCAATAGATGTGGGACACATGTGCGAGGACAC
AATCACCTACGAAT GCCCTAAACTTGATGCTGGTAATGACCCAGAGGA
CATTGACTGTTGGTGCGACAAACAAGCCGTGTATGTCCAGTATGGGCG
TTGCACGAGGACCAGGCACTCCAGGAGAAGTAGAAGATCTGTGTCAGT
GCAAACCCACGGAGAAAGCTCCCTAGTGAACAAAAAAGAAGCTTGGA
TGGATTCGACGAAAGCCACGCGGTATCTCATGAAAACAGAAAATTGGA
TCATACGGAATCCAGGCTATGCTCTCGTGGCAGTGGCACTCGGATGGA
TGCTTGGCAGCAACAACGGCCAGCGTGTGGTGTTCACAATTCTCTTGTT
GTTGGTCGCACCCGCATACAGC3'

***Nucleotide Sequence of E Gene of the Muar Strain of JEV***
***(From 978 to 2477)***

5' TTTAACTGCCTAGGCATGGGCAACCGCGACTTCATTGAAGGAGTCAG
CGGAGCCACGTGGGTAGACCTGGTGCTGGAAGGAGACAGTTGCCTCAC
CATCATGGCGAACGATAAACCAACACTGGACGTGCGCATGATAAACAT

TGAAGCCACGCAACTGGCTGAAGTACGAACCTATTGCTACCACGCTAC
AGTGGCTGACATTTCAACAGTAGCAAGATGCCCCACGACTGGAGAAGC
CCACAACACAAGACGAGCCGATAGCAGTTATGTTTGCAAGCAAGGCTA
TACAGACCGTGGATGGGGAAACGGATGCGGGTTGTTTGGGAAAGGCA
GCATTGACACATGCGCTAAATTTGTCTGCAGCCACAAGGCCATTGGGA
AGATAATACAGCCAGAAAATATCAAATATGAAGTTGGAGTATTTGTCC
ATGGAACCACAACAGCCGAGAACCATGGAAACTACTCCGCTC AGATT
GGAGCTTCCCAGGCTGCCAAGTTCACCATCACGCCCAATGCTCCTTCC
ATCACCCTGAAGCTTGGGGACTAC GGAGAAGTCACAATGGATTGCGA
GCCTCGTAGTGGATTTAACACTGAAGCATTTTATGTGCTGACCGTTGG
GACTAAGTCGTTTCTAGTCCATCGCGAATGGTTTAATGATTTGGCGCTT
CCATGGCTGTCTCCATCTAGCACAAACTGGAGAAACAGAGAGATCTTG
CTGGAATTTGAAGAAGCCCACGCGACGAAACAGTCTGTTGTTGCACTT
GGATCACAAGAGGGAGCTCTACACCAGGCTCTGGCTGGCGCCATAGTG
GTGGAGTATTCTAGCTCAGTGAAGTTAACTTCTGGCCACCTCAAATGT
AGACTAAAAATGGACAAGTTGGCCTTGAAAGGAACCACCTATGGCATG
TGCACAGAGAAGTTCTCCTTTTCGAAAAACCCAGCTGACACTGGTCAT
GGCACGGTCGTCATAGAATTGCAGTACACTGGCACTGATGGACCGTGC
AAGATACCCATCTCTTCAGTGGCCAGCCTGAATGATTTGACTCCAGTT
GGCAGATTGGTGACAGTCAATCCTTTTGTTGCCACATCCACTGCCAACT
CGAAAGTTTTGGTGGAACTTGAACCACCGTTTGGAGATTCATTCATTGT
TGTTGGGAGAGGAGACAAGCAGATTAACCACCATTGGCACAAGGCAG
GCAGTTCGCTGGGAAAGGCTTTTACCACTACCCTGAAAGGTGCCCAGA
GGTTAGCTGCCCTTGGCGACACGGCCTGGGATTTTGGGTCCATTGGAG
GAGTTT TTAATTCCATTGGCAAGGCCGTGCACCAGGTGTTTGGAG GAG
CTTTTAGAACACTTTTTGGTGGCATGTCTTGGATAACACAAGGATTGAT
GGGAGCACTGCTGCTGTGGATGGGTATCAATGCGCGAGACCGGTCGAT
CGCACTGGCCTTTCTTGCTACAGGAGGCGTGCTCTTGTTTCTGGCTACC
AATGTCCACGCT3'

## ***Nucleotide Sequence of NS1 Gene of the Muar Strain of JEV (From 2478 to 3533)***

5' GACACTGGCTGCGCCATTGATGTGACTAGGAAAGAAATGAGGTGCG
GCAGTGGCATATTTGTGCACAATGATGTGGAGGCTTGGGTGGACAGAT
ACAAGTATTTGCCTGAGACCCCCAAGTCTCTGGCCAAAATAGTTCACA
AAGCACATAAGGA AGGCATTTGCGGAGTGAGATCAGTCACCAGGCTG
GAACATCAAATGTGGGAAGCCGTCAGAGATGAGTTAAATGTCCTACTG
AAGGAGAACGCAGTAGATCTTAGTGTGGTGGTGGACAAACCAGTGGG
AAGATACCGACCAGCGCCACTGCGGCTATCCATGACCCAGGAAAAGTT
TGAGATGGGTTGGAAAGCAT GGGGGAAGAGCATTCTCTTTGCACCAGA

ACTAGCCAATT CAACATTTGTGATTGACGGACCCGAAACCAAAGAGTG
TCCAGACGAGCGCAGAGCATGGAACAGCATGCAGATTGAAGACTTTGG
GTTTGGCATCACGTCGACTCGAGTGTGGTTGAAGATCAGGGAGGAGCG
CACGGATGAATGTGATGGCGCCATCATTGGCACGGCCGTCAAAGGAAA
TATGGCGGTGCACAGTGACTTGTCATACTGGATTGAAAGCCATC TCAA
TGACACCTGGAAACTTGAAAGAGCCGTGTTTGGAGAGATTAAATCGTG
CACCTGGCCAGAAACACACACGCTTTGGGGAGATGGTGTTGAGGAAAG
TGAGTTAATAATACCACACACGCTCGCAGGACCCAAAAGTAAGCACAA
TAGAAGAGAGGGGTATAAAACACAGAACCAAGGACCATGGGATGAGAG
TGAAATCACCCT T GACTTTGACTACTGTCCGGGGACAACAGTTACCAT
TGC TGAAGGATGTGGAAAAAGAGGACCCTCAATCAGAACCACCACTG
ACAGCGGGAAATTAATCACTGATTGGTGCTGTAGGAGCTGTACTTTGC
CCCCGCTGAGATTTAGAACAGCCAGTGGCTGCTGGTATGGAATGGAAA
TCCGGCCCATGAAGCATGACGAATCCACGCTTGTGAAGTCACAAGTCA
ATGCA3'

## ***Nucleotide Sequence of NS2 Gene of the Muar strain of JEV (From 3534 to 4607)***

5' TTCAATGGGGAGATGATTGATCCTTTTCAGTTGGGCCTTCTGGTGATC
TTTCTGGCCACCCAGGAGGTCCTTCGCAAGAGGTGGACGGCCAGACTA
ACGATCCCTGCGGTTTTGGGGGCCCTACTTGTTCTGATGCTTGGGGGCA
TCACCTACACTGATCTGGTGAGATATGTGGTACTAGTGGCTGCTGCCTT
CGCTGAAGCTAACAATGGAGGAGATGTGGTCCACTTGGCTCTGATCGC
CGTTTTTAAGATTCAGCCGGCATTCCTAGTCATG AGCATAGCAAGTAC
CAATTGGACTAACCAGGAGAACATTGCTTTAGTGCTAGGAGCTGCTTT
CTTTCAGATGGCTTCAA CGGACTTGGAGTTTGGCATCCATGGGTTGCT
GAACGCAGCGGCGACGGCCTGGATGGTGGTGCGGGCGATTACGTTCCC
CACGACCTCCACCATCACGATGCCCATTCTAGCTTTGTTGGCACCAGG
AATGAGAGCTCTTCATCTCGACACCTACAGAATTTTTCTGCTCATCATT
GGAGTCTGTGCTCTGCTGCATGAAAGGAAGAAAACTATGGCAAAAAAG
AAAGGTGCTGTCCTCTTAGGCCTGGCCCTCAGTTCTACTGGGTGGTTTT
CACCAGCCATTATGGCTGCTGGGCTCATGGCTTGCAACCCAAACAAGA
AAAGAGGATGGCCAGCGACAGAATTCCTGTCTGCAATTGGGCTAATGT
TTGCCATTGTTGGGGGTCTGGCCGAGTTGGACATTGACTCCATGGCAAT
ACCTTTTATGTTAGCTGGACTTATGGCAGTGTCGTATGTGGTGTCAGGA
AAAGCAACAGACATGTGGTTGGAACGCGCAGCCGACATTAGTTGGGAA
GTGGACGCCGCGATCACAGGTAGCAGCCAGAGGTTGGATGTCAAATTG
GATGACGATGGAGATTTCCATCTTATTGATGACCCAGGCGTCCCATGG
AAAATTTGGGTATTGCGCATGTCTTGTATAGGATTGGCCGCCTTCACAC

CATGGGCCATTATACCAGCAGCTTTTGGATACTGGCTGACCCTGAAAA
CCACGAAGAGG3'

## ***Nucleotide Sequence of NS3 Gene of the Muar Strain of JEV (From 4608 to 6464)***

5' GGAGGCGTCTTCTGGGACACACCATCTCCCAAAGTCTACGCAAAAGG
AGATACAACCACAGGAGTGTACAGGATAATGGCGCGAGGGATCCTTGG
CGTCTACCAAGCAGGCGTCGGAGTGATGTATGAGAACGTGTTCCACAC
TCTCTGGCACACGACTAGAGGAGCCGCCATAATGAGTGGTGAAGGGAA
ACTAACACCGTACTGGGGAAGTGTCAAGGAAGACCGCATAACTTATGG
GGGTCCATGGAGATTCGACCGAAAATGGAATGGAGTGGATGACGTGCA
GATGATTGTCGTTGAACCAGGGAAGGCAGCCGTGAACGTCCAAACAAA
ACCAGGAGTGTTCCGGACCCCGCACGGAGAGATCGGAGCTGTCAGCTT
AGATTATCCTAGTGGGACATCAGGCTCACCCATCCTGGACGTCAACGG
TGACATTATTGGATTGTATGGAAACGGAGTTGAACTTGGAGATGGCTC
ATATGTAAGCGCCATTGTGCAGGGTGAACGTCAAGAGGAACCCGTCCC
TGATGCATACAATCCAAACATGCTCAAGAAAAGGCAGCTGACAGTGTT
GGACCTGCATCCAGGATCGGGCAAAACAAGGAAAATTTTGCCCCAAAT
CATCAGGGATGCTATTCAACAACGCCTCAGAACAGCTGTTTTGGCACC
CACTCGTGTCGTCGCGGCAGAGATGGCAGAAGCTCTGAGAGGACTCCC
CGTCAGATACCAAACTTCAGCGGTCCAGCGGGAACACCAGGGAAATGA
GATAGTTGATGTCATGTGTCATGCCACTCTAACGCATAGACTGATGTC
ACCAAACCGCGTTCCCAATTACAACTTGTTCGTTATGGATGAGGCTCA
CTTCACTGACCCAGCTAGCATTGCTGCAAGAGGATACATATCTACCAA
AGTGGAATTGGGAGAAGCTGCGGCTATTTTTATGACTGCCACTCCACC
CGGAACGACTGACCCGTTCCCGGACTCCAATGCTCCCATTCATGATTT
GCAGGACGAAATCCCTGACAGAGCATGGAGCAGTGGGTATGAATGGA
TAACTGAGTATGTGGGCAAGACAGTATGGTTTGTGGCGAGCGTAAAAA
TGGGCAATGAAATCGCAGTGTGCTTACAGAGAGCTGGGAAGAGGGTCA
TCCAGTTGAATCGGAA ATCCTATGACACCGAGTACCCTAAATGTAAGA
ATGGGGATTGGGATTTTGTCATCACCACGGACATTTCTGAGATGGGGG
CCAACTTCGGAGCGAGCAGAGTGATTGATTGTAGGAAAAGTGTGAAAC
CCACCATTTTGGAGGAGGGAGAAGGAAGAGTCATTCTCAGTAATCCAT
CGCCCATCACCAGTGCGAGCGCAGCTCAGCGGAGAGGCAGAGTGGGC
AGAAATCCAAATCAGGTTGGGGATGAGTACCATTATGGAGGTACCACG
AGTGAGGATGACACCAACCTAGCCCACTGGACAGAAGCCAAGATCATG
CTTGATAACATCCACTTGCCAAATGGATTAGTAGCTCAACTTTATGGA
CCTGAAAGGGAGAAGGCCTTCACAATGGATGGTGAGTATCGATTGAGG
GGTGAGGAAAAGAAAAATTTTCTGGAGCTGATTAGAACAGCCGACCTC
CCAGTATGGCTGGCCTACAAAGTGGCGTCAAATGGAATACAATACACC

GATCGGAGATGGTGTTTTGATGGTCCCCGGACGAATGCCATCTTAGAG
GACAGCACTGAAGTGGAGATAATCACCAGAATGGGAGAGAGAAAAAC
TCTAAAACCAAGATGGCTGGACGCACGTGTGTATGCGGATCACCAGGC
TCTGAAGTGGTTCAAGGACTTCGCGGCAGGGAAGAGA3'

## *Nucleotide Sequence of NS4 Gene of the Muar Strain of JEV (From 6465 to 7679)*

5' TCAGCTGTCAGCTTTCTAGAGGTGCTTGGGCGCATGCCGGAACATTT
CATGGGGAAAACTCGTGAAGCCCTTGACACAATGTACCTAGTTGCCAC
AGCAGAGAAAGGGGGGAAAGCCCATCGAATGGCTCTAGAAGAATTGC
CAGATGCACTGGAAACGGTGACACTCATTGCAGCGATAGCCGTGATGA
CAGGTGGGTTCTTCTTGCTCATGATGCAACGAAAGGGGATAGGGAAAA
TGGGCCTGGGCGCCCTTGTGCTCACCCTGGCCACCTTCTTCTTGTGGAT
GGCAGAGGTATCAGGGACGAAAATAGCCGGAACCCTACTCATAGCACT
GTTGCTCATGGTGGTACTCATTCCGGAGCCGGAGAAACAAAGATCCCA
AACGGACAATCAGTTGGCCGTGTTCCTGATCTGCGTCCTAACTGTGGT
AGGAATCGTGGCTGCTAATGAATATGGTATGCTTGAGAAGACAAAGGA
AGACATAAGGAGCATCCTTGGCAACAGGGCTCAGACATCCAGCGTGCC
TGGAAGTCTGTCAAGCCTGGCGCTCGATTTGCGACCAGCGACAGCTTG
GGCTCTATACGGAGGCAGCACAGTGGTTTTAACTCCACTGCTGAAACA
CTTGATCACTTCTGAGTATGTGACAACATCTCTAGCTTCAATCAACTCA
CAGGCCGGCTCACTCTTTGTTCTACCAAGAGGCATGCCCTTCACAGATT
TGGATCTGACGGTTGGACTCGTCTTTCTGGGCTGCTGGGGGCAAGTCA
CTCTTACCACTTTTTTGACAGCTGGAGTGCTGGCAGTTTTGCACTACGG
CTACATGCTCCCTGGGTGGCAAGCCGAAGCTTTGAGGGCAGCTCAAAG
AAGAACAGCCGCGGGCATCATGAAGAACGCCGTTGTGGATGGGATGGT
GGCCACTGACGTGCCAGAACTGGAAAG AACAACACCTCTAATGCAGA
AAAAGGTGGGGCAAGTGTTGCTAATAGGAGTAAGCATAGCTGCTTTTC
TCGTCAACCCTAATGTCACAACAGTACGAGAGGCCGGTGTGTTGGTGA
CCGCCGCCACGCTCACCCTATGGGACAACGGAGCAAGTGCTGTTTGGA
ATTCAACTACAGCCACAGGACTCTGCCACGTTATGCGAGGCAGCTACT
TGGCTGGTGGTTCAATAGCCTGGACCCTCATTAAAAATGTTGATAAAC
CATCTCTGAAAAGA3'

## *Nucleotide Sequence of NS5 Gene of the Muar Strain of JEV (From 7680 to 10.394)*

5' GGAAGACCTGGAGGAAGAACGCTGGGTGAGCAATGGAAAGAAAGGT
TGAACGCCATGAACAAGGAAGAGTTTTTTAAGTACAGGAAAGAAGCCA
TAGTCGAGGTGGACCGCACAGAGGCACGCAGGGCTAGACGAGAGAAC

AACAAAGTGGGAGGCCATCCCGTGTCACGAGGATCAGCAAAGCTCCGA
TGGATAGTGGAGAAAGGGTTTGTCTCACCAGTTGGAAAGGTCGTAGAT
CTTGGTTGCGGGCGGGGAGGTTGGTGCTATTATACAGCCACCCTGAAA
AAAGTCCAGGAAGTCAAGGGTTACACAAAAGGAGGGGCTGGACATGA
GGAACCTATGTTGATGCAAAGTTACGGCTGGAATTTGGTCACAATGAA
GAGTGGAGTGGACGTGTTCTACAGACCTTCAGAGCCCAGTGACACCCT
GCTCTGTGATATAGGGGAGTCTTCCCCAAGTCCTGACGTCGAAGAACA
ACGCACTTTGCGAGTTTTGGAAATGGCATCAGAGTGGTTGCACCGAGG
GCCCAGGGAATTTTGCATAAAAGTCCTATGTCCATACATGCCAAAGGT
GATAGAAAAGATGGAAACACTGCAACGCCGCTTTGGAGGTGGACTGGT
GCGTGTTCCTCTGTCACGCAATTCGAACCATGAGATGTACTGGGTCAG
TGGAGCCGCTGGGAATGT GGTACACGCTGTAAACATGACCAGTCAAGT
CTTGCTAGGGCGAATGGACCGAGCAGTCTGGAGAGGACCCAAATATGA
GGAAGATGTTAACTTGGGAAGCGGGACTAGAGCTGTAGGAAAAGGTG
AGGTTCACAGTGACCAAGGGAAAATAAAAAAGCGGATAGAGAAACTG
AAAGATGAGTACGCAGCAACCTGGCATGAGGATCCAGAACACC CATA
CCGCACCTGGACATACCATGGAAGTTACGAAGTGAAAGCCACCGGGTC
AGCCAGCTCCCTTGTCAATGGAGTGGTAAAACTCATGAGCAAGCCTTG
GGACGCCATCACCAGTGTCACCACTATGGCCATGACTGATACTACTCC
TTTTGGTCAGCAGAGAGTTTTCAAAGAAAAGGTTGACACTAAAGCGCC
TGAACCACCTGCAGGAGTCCGGGAAGTGCTGGACGAGACGACCAATTG
GCTGTGGGCCTACCTATCAAGAGAGAAAAAACCTCGTTTGTGCACGAG
AGAGGAATTTGTTCGGAAAGTCAACAGCAACGCGGCTCTTGGGGCCAT
GTTTGCCGAGCAGAACCAATGGAGCTCAGCCAGAGAGGCTGTTAGCGA
CCCGGCCTTCTGGGACATGGTTGACGTTGAAAGAGAGAACCACCTACG
AGGGGAGTGCCATACTTGCATCTATAACATGATGGGGAA AAGAGAAA
AGAAACCCGGTGAGTTTGGGAAGGCCAAGGGAAGCAGGGCCATCTGG
TTCATGTGGCTT GGAGCTCGCTACCTGGAATTTGAGGCACTTGGGTTC
TTGAACGAGGACCATTGGTTGAGTAGGGAGAATTCAGGAGGAGGAGT
GGAAGGCTCAGGCATACAGAAGCTAGGGTACATCCTGCGAGACATCTC
AATGAAAGCTGGAGGAAAAATGTATGCTGATGACACAGCTGGCTGGG
ACACTAGGATCACAAGGGTTGATCTGGACAATGAGGCGAAGGTACTAG
AGCTCTTGGATGGGGAACATAGGATGTTGGCCCGTGCCATAATAGAAT
TGACCTACAAACACAAAGTTGTCAAAGTGATGAGGCCAGCAGCCGGTG
GAAAGACTGTAATGGATGTGATCTCCAGAGAAGACCAAAGAGGGAGT
GGACAAGTGGTTACATATGCTCTTAACACCTTCACAAACATAGCCGTC
CAACTGGTAAGGTTAATGGAGGCAGAAGG AGTTGTTGGCCCGCAGGA
CGTAGAACAGCTCCCAAGAAAAACCAAGTTTGCAGTCAGGACATGGCT
TTTTGAAAATGGAGAGGAGAGAGTCACCAGAATGGCAGTAAGTGGGG
ATGATTGTGTTGTCAAACCACTCGATGACAGATTCGCGAATGCTCTGC

ATTTCTTGAATGCGATGTCAAAGGTGAGGAAAGACATACAGGAATGGA
AACCATCTCAAGGCTGGCATGACTGGCAGCAAGTCCCTTTCTGCTCAA
ATCATTTCCAGGAGATCGTGATGAAAGATGGCAGAAGCCTCGTCGTGC
CCTGCCGGGGACAGGATGAATTAATAGGCAGAGCCCGGATTTCACCA
GGAGCAGGATGGAACGTGAGAGACACGGCCTGCTTAGCCAAGGCATA
CGCCCAAATGTGGCTCCTCCTCTATTTCCACCGGAGAGACCTGCGCCTC
ATGGCCAACGCAATCTGTTCAGCTGTTCCAGTAGACTGGGTGCCCACA
GGCCGGACTTCATGGTCGATACACTCAAAAGGAGAATGGATGACAACA
GAAGACATGTTGCAGGTGTGGAACAGAG TATGGATTGAAGAGAATGA
ATGGATGAGAGACAAAACTCCCGTCGCCAGTTGGACCGACGTTCCTTA
CGTCGGGAAAAGGGAAGACATCTG GTGTGGCAGCTTGATCGGAACGC
GAACAAGAGCTACCTGGGCAGAGAACATCTATGCAGCAATCAACCAA
GTGAGGGCAATAATTGGAAACGAAAAGTATGTGGACTACATGACATCA
CTCAGGAGGTATGAAGACACTTTGGTCCAGGAAGATAGAGTCATT **3'**

## ***Nucleotide Sequence of 3' NCR of the Muar Strain of JEV (From 10.395 to 10.988)***

**5'** TAAAGAACTCTTGAAAACAAATGTAAATAGTAGTAATTGTTTAGTGT
AAATAGTGTAAATAAATAAATTTAGATAGGAAGTCAGGCCGACGCGAG
TCGCCACCGGAAGCTGAGTAGACGGTGCTGCCTGCGCCTCAGCCCCAG
GAGGACTGGGTTAACAAATCTGACAACCGAAGGTAGGAAAGCCCTCA
GAACCGTCTCGGAAGAAGGTCCCTGCTTACTGGAGGTTGGAAGACCGT
GTCAGGCCACGTAAGTGCCACTTCGCTGAGGAGTGCAGCCTGTACAGC
CCCGGGAGGACCGGGTAAACAAAGCCGAAAAGGCCCCCACGGCCCAA
ACCTCATCTAGGATGCAATAGATGAGGCGTAAGGACTAGAGGTTAGAG
GAGACCCCGTGGAAAAGAAGATGCGGCCCAAACTCTTTCGAAGCTGTA
GAAGGAGTGGAAGGACTAGAGGTTAGAGGAGACCCCGCATTTGCATC
AAAACAGCATATTGACACCTGGGATTAGACTAGGAGATCTTCTGATCT
ATCTCAACATCAGCTACAAGGCACCGAGCGCCGAAGTATGTAGCTGGT
GGTGGGGAAGAACACAGGATCT**3'**

# APPENDIX B

## *Amino Acid Sequence of C Protein of the Muar Strain of JEV*

5' MTKKPGGPGRNRAINMLKRGLPRVSPLVGVKRVIMNLLDGRGPIRFVL
ALLAFFKFTALAPTKALISRWKAVEKSVAMKHLTSFKKELGTLINAVNK
RGKKQNKRGGSNGTIIWMIGLAVVFATVSA3'

## *Amino Acid Sequence of prM Protein of the Muar Strain of JEV*

5' VKLSNFQGKVLMTINNTDVADVITIPTSKGTNRCWVRAIDVGHMCEDT
ITYECPKLDAGNDPEDIDCWCDKQAVYVQYGRCTRTRHSRRSRRSVSVQ
THGESSLVNKKEAWMDSTKATRYLMKTENWIIRNPGYALVAVALGWML
GSNNGQRVVFTILLLLVAPAYS3'

## *Amino Acid Sequence of E Glycoprotein of the Muar Strain of JEV*

5' FNCLGMGNRDFIEGVSGATWVDLVLEGDSCLTIMANDKPTLDVRMINI
EATQLAEVRTYCYHATVADISTVARCPTTGEAHNTRRADSSYVCKQGYT
DRGWGNGCGLFGKGSIDTCAKFVCSHKAIGKIIQPENIKYEVGVFVHGTT
TAENHGNYSAQIGASQAAKFTITPNAPSITLKLGDYGEVTMDCEPRSGFN
TEAFYVLTVGTKSFLVHREWFNDLALPWLSPSSTNWRNREILLEFEEAHA
TKQSVVALGSQEGALHQALAGAIVVEYSSSVKLTSGHLKCRLKMDKLAL
KGTTYGMCTEKFSFSKNPADTGHGTVVIELQYTGTDGPCKIPISSVASLN
DLTPVGRLVTVNPFVATSTANSKVLVELEPPFGDSFIVVGRGDKQINHHW
HKAGSSLGKAFTTTLKGAQRLAALGDTAWDFGSIGGVFNSIGKAVHQVF
GGAFRTLFGGMSWITQGLMGALLLWMGINARDRSIALAFLATGGVLLFL
ATNVHA3'

## *Amino Acid Sequence of NS1 Protein of the Muar Strain of JEV*

5' DTGCAIDVTRKEMRCGSGIFVHNDVEAWVDRYKYLPETPKSLAKIVHK
AHKEGICGVRSVTRLEHQMWEAVRDELNVLLKENAVDLSVVVDKPVGR
YRPAPLRLSMTQEKFEMGWKAWGKSILFAPELANSTFVID GPETKECPD
ERRAWNSMQIEDFGFGITSTRVWLKIREERTDECDGAIIGTAVKGNMAVH
SDLSYWIESHLNDTWKLERAVFGEIKSCTWPETHTLWGDGVEESELIIPH
TLAGPKSKHNRREGYKTQNQGPWDESEITLDFDYCPGTTVTIAEGCGKR
GPSIRTTTDSGKLITDWCCRSCTLPPLRFRTASGCWYGMEIRPMKHDEST
LVKSQVNA3'

## *Amino Acid Sequence of NS2 Protein of the Muar Strain of JEV*

5' FNGEMIDPFQLGLLVIFLATQEVLRKR WTARLTIPAVLGALLVLMLGGI
TYTDL VRYVVLVAAAFAEANNGGDVVHLALIAVFKIQPAFLVMSIASTN
WTNQENIALVLGAAFFQMASTDLEFG I HGLLNAAATAWMVVRAITFPTT
STITMPILALLAPGMRALHLDTYRIFLLIIGVCALLHERKKTMAKKKGAV
LLGLALSSTGWFSPAIMAAGLMACNPNKKRGWPATEFLSAIGLMFAIVG
GLAELDIDSMAIPFMLAGLMAVSYVVSGKATDMWLERAADISWEVDAAI
TGSSQRLDVKLDDDGDFHLIDDPGVPWKIWVLRMSCIGLAAFTPWAIIPA
AFGYWLTLKTTKR3'

## *Amino Acid Sequence of NS3 Protein of the Muar Strain of JEV*

5' GGVFWDTPSPKVYAKGDTTTGVYRIMARGILGVYQAGVGVMYENVFH
TLWHTTRGAAIMSGEGKLTPYWGSVKEDRITYGGPWRFDRKWNGVDDV
QMIVVEPGKAAVNVQTKPGVFRTPHGEIGAVSLDYPSGTSGSPILDVNGD
IIGLYGNGVELGDGSYVSAIVQGERQEEPVPDAYNPNMLKKRQLTVLDL
HPGSGKTRKILPQIIRDAIQQRLRTAVLAPTRVVAAEMAEALRGLPVRYQ
TSAVQREHQGNEIVDVMCHATLTHRLMSPNRVPNYNLFVMDEAHFTDPA
SIAARGYISTKVELGEAAAIFMTATPPGTTDPFPDSNAPIHDLQDEIPDRA
WSSGYEWITEYVGKTVWFVASVKMGNEIAVCLQRAGKRVIQLNRKSYD
TEYPKCKNGDWDFVITTDISEMGANFGASRVIDCRKSVKPTILEEGEGRV
ILSNPSPITSASAAQRRGRVGRNPNQVGDEYHYGGTTSEDDTNLAHWTE
AKIMLDNIHLPNGLVAQLYGPEREKAFTMDGEYRLRGEEKKNFLELIRTA
DLPVWLAYKVASNGIQYTDRRWCFDGPRTNAILEDSTEVEIITRMGERKT
LKPRWLDARVYADHQALKWFKDFAAGKR3'

## *Amino Acid Sequence of NS4 Protein of the Muar Strain of JEV*

5' SAVSFLEVLGRMPEHFMGKTREALDTMYLVATAEKGGKAHRMALEEL
PDALETVTLIAAIAVMTGGFFLLMMQRKGIGKMGL GALVLTLATFFLWM
AEVSGTKIAGTLLIALLLMVVLIPEPEKQRSQTDNQLAVFLICVLTVVGIV
AANEYGMLEKTKEDIRSILGNRAQTSSVPGSLSSLALDLRPATAWALYGG
STVVLTPL LKHLITSEYVTTSLASINSQAG SLFVLPRGMPFTDLDLTVGL
VFLGCWGQVTLTTFLTAGVLAVLHYGYMLPGWQAEALRAAQRRTAAGI
MKNAVVDGMVATDVPELERTTPLMQKKVGQVLLIGVSIAAFLVNPNVTT
VREAGVLVTAATLTLWDNGASAVWNSTTATGLCHVMRGSYLAGGSIAW
TLIKNVDKPSLKR3'

## *Amino Acid Sequence of NS5 Protein of the Muar Strain of JEV*

5' GRPGGRTLGEQWKERLNAMNKEEFFKYRKEAIVEVDRTEARRARREN
NKVGGHPVSRGSAKLRWIVEKGFVSPVGKVVDLGCGRGGWCYYTATLK
KVQEVKGYTKGGAGHEEPMLMQSYGWNLVTMKSGVDVFYRPSEPSDTL
LCDIGESSPSPDVEEQRTLRVLEMASEWLHRGPREFCIKVLCPYMPKVIE
KMETLQRRFGGGLVRVPLSRNSNHEMYWVSGAAGNVVHAVNMTSQVLL
GRMDRAV WRGPKYEEDVNLGSGTRAVGKGEVHSDQGKIKKRIEKLKDE
YAATWHEDPEHPYRTWTYHGSYEVKATGSASSLVNGVVKLMSKPWDAI
TSVTTMAMTDTTPFGQQRVFKEKVDTKAPEPPAGVREVLDETTNWLWA
YLSREKKPRLCTREEFVRKVNSNAALGAMFAEQNQWSSAREAVSDPAFW
DMVDVERENHLRGECHTCIYNMMGKREKKPGEFGKAKGSRAIWFMWLG
ARYLEFEALGFLNEDHWLSRENSGGGVEGSGIQKL GYILRDISMKAGGK
MYADDTAGWDTRITRVDLDNEAKVLELLDGEHRMLARAIIELTYKHKVV
KVMRPAAGGKTVMDVISREDQRGSGQVVTYALNTFTNIAVQLVRLMEA
EGVVGPQDVEQLPRKTKFAVRTWLFENGEERVTRMAVSGDDCVVKPLD
DRFANALHFLNAMSKVRKDIQEWKPSQGWHDWQQVPFCSNHFQEIVMK
DGRSLVVPCRGQDELIGRARISPGAGWNVRDTACLAKAYAQMWLLLYF
HRRDLRLMANAICSAVPVDWVPTGRTSWSIHSKGEWMTTEDMLQVWNR
VWIEENEWMRDKTPVASWTDVPYVGKREDIWCGSLIGTRTRATWAENIY
AAIN QVRAII GNEKYVDYMTSLRRYEDTLVQEDRVI3'

## APPENDIX C

The differences in the nucleotide sequence of the three structural genes (C, prM and E) between Muar (A), sequenced by Hasegawa et al. (1994), and Muar (B) sequenced by me. (different nucleotides are underlined)

```
Muar(A)    ATGACTAAAA AACCAGGAGG GCCCGGTAGA AACCGGGCTA TCAATATGCT
Muar(B)    ATGACTAAAA AACCAGGAGG GCCCGGTAGA AACCGGGCTA TCAATATGCT

Muar(A)    GAAACGCGGT TTACCCCGCG TATTCCCACT TGTGGGGGTG AAGAGGGTAA
Muar(B)    GAAACGCGGT TTACCCCGCG TATCCCCACT TGTGGGGGTG AAGAGGGTAA

Muar(A)    TAATGAACTT GCTGGACGGC AGAGGGCCAA TACGATTCGT TTTGGCTCTC
Muar(B)    TAATGAACTT GCTGGACGGC AGAGGGCCAA TACGATTCGT TTTGGCTCTC

Muar(A)    TTGGCGTTTT TCAAGTTCAC AGCATTGGCC CCAACTAAGG CACTTATTAG
Muar(B)    TTGGCGTTTT TCAAGTTCAC AGCATTGGCC CCAACTAAGG CACTTATTAG

Muar(A)    CCGATGGAAA GCAGTAGAGA AGAGCGTCGC GATGAAACAC CTCACCAGCT
Muar(B)    CCGATGGAAA GCAGTAGAGA AGAGCGTCGC GATGAAACAC CTCACCAGCT

Muar(A)    TCAAAAGAGA ACTGGGAACG CTCATCAACG CTGTGAATAA GAGGGGCAAA
Muar(B)    TCAAAAAGGA ACTGGGAACG CTCATCAACG CTGTGAATAA GAGGGGCAAA

Muar(A)    AAACAAAACA AAAGAGGAGG AAATAATGGC TCAATTATTT GGATCGTAGG
Muar(B)    AAACAAAACA AAAGAGGAGG AAGTAATGGA ACAATTATTT GGATGATAGG

Muar(A)    TTTGGCAGTC GTCATAGCAT GCGCAGGAGC AATGAAGCTG TCGAATTTCC
Muar(B)    TTTGGCAGTC GTGTTCGCCA CTGTGAGTGC AGTCAAGCTG TCAAACTTTC

Muar(A)    AGGGGCAGCT GCTGATGACC ATCAACAACA CGGACATTGC TGATGTGATC
Muar(B)    AGGGCAAGGT GCTGATGACA ATCAATAACA CCGACGTGGC TGATGTGATC

Muar(A)    GTGATTCCCA CCTCGAAAGG AGAGAATAGA TGTTGGGTTC GGGCAATAGA
Muar(B)    ACCATTCCCA CCTCGAAAGG GACCAATAGA TGTTGGGTTC GGGCAATAGA

Muar(A)    TGTCGGCAAC ATGTGTGAGG ACACAATCAC CTACGAATGC CCTAAGCTCA
Muar(B)    TGTGGGACAC ATGTGCGAGG ACACAATCAC CTACGAATGC CCTAAACTTG

Muar(A)    CCATGGGTAA TGACCCAGAG GACATTGACT GTTGGTGCGA CAAACAAGCC
Muar(B)    ATGCTGGTAA TGACCCAGAG GACATTGACT GTTGGTGCGA CAAACAAGCC

Muar(A)    GTCTATGTCC AGTATGGGCG GTGCACGAGG ACCAGGCACT CCAGGAGAAG
Muar(B)    GTGTATGTCC AGTATGGGCG TTGCACGAGG ACCAGGCACT CCAGGAGAAG
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Muar(A)          TAGAAGATCT GTGTCAGTGC AAACCCACGG AGAAAGCTCC CTAGTGAACA
Muar(B)          TAGAAGATCT GTGTCAGTGC AAACCCACGG AGAAAGCTCC CTAGTGAACA

Muar(A)          AAAAAGAAGC TTGGATGGAT TCGACGAAAG CCACGCGGTA TCTCATGAAA
Muar(B)          AAAAAGAAGC TTGGATGGAT TCGACGAAAG CCACGCGGTA TCTCATGAAA

Muar(A)          ACAGAAAATT GGATCATACG GAATCCAGGC TATGCTCTCG TGGCAGTGGC
Muar(B)          ACAGAAAATT GGATCATACG GAATCCAGGC TATGCTCTCG TGGCAGTGGC

Muar(A)          ACTCGGATGG ATGCTTGGCA GCAACAACGG CCAGCGTGTG GTGTTCACAA
Muar(B)          ACTCGGATGG ATGCTTGGCA GCAACAACGG CCAGCGTGTG GTGTTCACAA

Muar(A)          TTCTCCTGTT GTTGGTCGCA CCCGCATACA GCTTTAACTG CCTAGGCATG
Muar(B)          TTCTCTTGTT GTTGGTCGCA CCCGCATACA GCTTTAACTG CCTAGGCATG

Muar(A)          GGCAACCGCG ACTTCATTGA AGGAGTCAGC GGAGCCACGT GGGTAGACCT
Muar(B)          GGCAACCGCG ACTTCATTGA AGGAGTCAGC GGAGCCACGT GGGTAGACCT

Muar(A)          GGTGCTGGAA GGAGACAGTT GCCTCACCAT CATGGCGAAC GATAAACCAA
Muar(B)          GGTGCTGGAA GGAGACAGTT GCCTCACCAT CATGGCGAAC GATAAACCAA

Muar(A)          CACTGGACGT GCGCATGATA AACATTGAAG CCACGCAACT GGCTGAAGTC
Muar(B)          CACTGGACGT GCGCATGATA AACATTGAAG CCACGCAACT GGCTGAAGTA

Muar(A)          AGAACCTATT GCTACCACGC TACAGTGGCT GACATTTCAA CAGTAGCAAG
Muar(B)          CGAACCTATT GCTACCACGC TACAGTGGCT GACATTTCAA CAGTAGCAAG

Muar(A)          ATGCCCCACG ACTGGAGAAG CCCACAACAC AAGACGAGCC GATAGCAGTT
Muar(B)          ATGCCCCACG ACTGGAGAAG CCCACAACAC AAGACGAGCC GATAGCAGTT

Muar(A)          ATGTTTGCAA GCAAGGCTAT ACAGACCGTG GATGGGGAAA CGGATGCGGG
Muar(B)          ATGTTTGCAA GCAAGGCTAT ACAGACCGTG GATGGGGAAA CGGATGCGGG

Muar(A)          TTGTTTGGGA AAGGCAGCAT TGACACATGC GCAAAATTGT CCTGCAGCAG
Muar(B)          TTGTTTGGGA AAGGCAGCAT TGACACATGC GCTAAATTTG TCTGCAGCCA

Muar(A)          CAAAGCGATT GGGAAGATAA TACAGCCAGA AAATATCAAA TATGAAGTTG
Muar(B)          CAAGGCCATT GGGAAGATAA TACAGCCAGA AAATATCAAA TATGAAGTTG

Muar(A)          GCATATTTGT CCATGGAACC ACAACAGCCG AGAACCATGG AAATTACTCC
Muar(B)          GAGTATTTGT CCATGGAACC ACAACAGCCG AGAACCATGG AAACTACTCC

Muar(A)          GCTCAAGTTG GAGCTTCCCA GGCTGCAAAG TTCACCATCA CGCCCAATGC
Muar(B)          GCTCAGATTG GAGCTTCCCA GGCTGCCAAG TTCACCATCA CGCCCAATGC
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Muar(A)          TCCTTCCATC ACCCTGAAGC TTGGGGACTA CGGAGAAGTC ACAATGGATT
Muar(B)          TCCTTCCATC ACCCTGAAGC TTGGGGACTA CGGAGAAGTC ACAATGGATT

Muar(A)          GCGAGCCTCG TAGTGGATTT AACACTGAAG CATTTTATGT GCTGACCGTT
Muar(B)          GCGAGCCTCG TAGTGGATTT AACACTGAAG CATTTTATGT GCTGACCGTT

Muar(A)          GGGACTAAGT CGTTTCTAGT CCATCGCGAA TGGTTTAATG ATTTGGCGCT
Muar(B)          GGGACTAAGT CGTTTCTAGT CCATCGCGAA TGGTTTAATG ATTTGGCGCT

Muar(A)          CCCCTGGCTG TCTCCATCTA GCACAAACTG GAGAAACAGA GAGATCTTGC
Muar(B)          TCCATGGCTG TCTCCATCTA GCACAAACTG GAGAAACAGA GAGATCTTGC

Muar(A)          TGGAATTTGA AGAAGCCCAC GCGACGAAAC AGTCTGTTGT TGCACTTGGA
Muar(B)          TGGAATTTGA AGAAGCCCAC GCGACGAAAC AGTCTGTTGT TGCACTTGGA

Muar(A)          TCACAAGAGG GAGCTCTACA CCAGGCTCTG GCTGGCGCCA TAGTGGTGGA
Muar(B)          TCACAAGAGG GAGCTCTACA CCAGGCTCTG GCTGGCGCCA TAGTGGTGGA

Muar(A)          GTATTCTAGC TCAGTGAAGT TAACTTCTGG CCACCTCAAA TGTAGACTAA
Muar(B)          GTATTCTAGC TCAGTGAAGT TAACTTCTGG CCACCTCAAA TGTAGACTAA

Muar(A)          AAATGGACAA GTTGGCTCTG AAAGGAACCA CCTATGGCAT GTGCACAGAG
Muar(B)          AAATGGACAA GTTGGCCTTG AAAGGAACCA CCTATGGCAT GTGCACAGAG

Muar(A)          AAGTTCTCCT TTTCGAAAAA CCCAGCTGAC ACTGGTCATG GCACGGTCGT
Muar(B)          AAGTTCTCCT TTTCGAAAAA CCCAGCTGAC ACTGGTCATG GCACGGTCGT

Muar(A)          CATAGAATTG CAGTACACTG GCACTGATGG ACCGTGCAAG ATACCCATCT
Muar(B)          CATAGAATTG CAGTACACTG GCACTGATGG ACCGTGCAAG ATACCCATCT

Muar(A)          CTTCAGTGGC CAGCCTGAAT GATTTGACTC CAGTTGGCAG ATTGGTGACA
Muar(B)          CTTCAGTGGC CAGCCTGAAT GATTTGACTC CAGTTGGCAG ATTGGTGACA

Muar(A)          GTCAATCCTT TTGTTGCCAC ATCCACTGCC AACTCGAAAG TTTTGGTGGA
Muar(B)          GTCAATCCTT TTGTTGCCAC ATCCACTGCC AACTCGAAAG TTTTGGTGGA

Muar(A)          ACTTGAACCA CCGTTTGGAG ATTCATTCAT TGTTGTTGGG AGAGGAGACA
Muar(B)          ACTTGAACCA CCGTTTGGAG ATTCATTCAT TGTTGTTGGG AGAGGAGACA

Muar(A)          AGCAGATTAA CCACCATTTG CACAAGGTCG GAAGCACGCT GGGCAAGGCG
Muar(B)          AGCAGATTAA CCACCATTGG CACAAGGCAG GCAGTTCGCT GGGAAAGGCT

Muar(A)          TTCTCAACGA CGTTGAAAGG AGCTCAAAGA CTGGCTGTGT TGGGCGATAC
Muar(B)          TTTACCACTA CCCTGAAAGG TGCCCAGAGG TTAGCTGCCC TTGGCGACAC
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Muar(A)          GGACTGGGAC TTCGGCTCTA CTGGAGGGGT CTTCAATTCT ATAGGCAAGG
Muar(B)          GGCCTGGGAT TTTGGGTCCA TTGGAGGAGT TTTTAATTCC ATTGGCAAGG

Muar(A)          CCGTTCACCA CGTGTTCGGC GGTACCTTCA GAACCCTCTT TGGAGGAATG
Muar(B)          CCGTGCACCA GGTGTTTGGA GGAGCTTTTA GAACACTTTT TGGTGGCATG

Muar(A)          TCCTGGATAA CACAAGGACT AATGGGGGCT CTACTCCTCT GGATGGGGGT
Muar(B)          TCTTGGATAA CACAAGGATT GATGGGAGCA CTGCTGCTGT GGATGGGTAT

Muar(A)          CAAAGCACGA GACGGATCAA TCGCTTTAGC CTTTTTAGGC ACAGGGGGTG
Muar(B)          CAATGCGCGA GACCGGTCGA TCGCACTGGC CTTTCTTGCT ACAGGAGGCG

Muar(A)          TGCTTGTATT CTTGGCGACC AACGTGCATG CT
Muar(B)          TGCTCTTGTT TCTGGCTACC AATGTCCACG CT
```

# APPENDIX D

The differences in the amino acid sequence of the three structural genes (C, prM and E) between Muar (A), sequenced by Hasegawa et al. (1994), and Muar (B) sequenced by me. (different amino acids are underlined)

```
Muar (A)        MTKKPGGPGR NRAINMLKRG LPRVFPLVGV KRVIMNLLDG RGPIRFVLAL
Muar (B)        MTKKPGGPGR NRAINMLKRG LPRVSPLVGV KRVIMNLLDG RGPIRFVLAL

Muar (A)        LAFFKFTALA PTKALISRWK AVEKSVAMKH LTSFKRELGT LINAVNKRGK
Muar (B)        LAFFKFTALA PTKALISRWK AVEKSVAMKH LTSFKKELGT LINAVNKRGK

Muar (A)        KQNKRGGNNG SIIWIVGLAV VIACAGAMKL SNFQGQLLMT INNTDIADVI
Muar (B)        KQNKRGGSNG TIIWMIGLAV VFATVSAVKL SNFQGKVLMT INNTDVADVI

Muar (A)        VIPTSKGENR CWVRAIDVGN MCEDTITYEC PKLTMGNDPE DIDCWCDKQA
Muar (B)        TIPTSKGTNR CWVRAIDVGH MCEDTITYEC PKLDAGNDPE DIDCWCDKQA

Muar (A)        VYVQYGRCTR TRHSRRSRRS VSVQTHGESS LVNKKEAWMD STKATRYLMK
Muar (B)        VYVQYGRCTR TRHSRRSRRS VSVQTHGESS LVNKKEAWMD STKATRYLMK

Muar (A)        TENWIIRNPG YALVAVALGW MLGSNNGQRV VFTILLLLVA PAYSFNCLGM
Muar (B)        TENWIIRNPG YALVAVALGW MLGSNNGQRV VFTILLLLVA PAYSFNCLGM

Muar (A)        GNRDFIEGVS GATWVDLVLE GDSCLTIMAN DKPTLDVRMI NIEATQLAEV
Muar (B)        GNRDFIEGVS GATWVDLVLE GDSCLTIMAN DKPTLDVRMI NIEATQLAEV

Muar (A)        RTYCYHATVA DISTVARCPT TGEAHNTRRA DSSYVCKQGY TDRGWGNGCG
Muar (B)        RTYCYHATVA DISTVARCPT TGEAHNTRRA DSSYVCKQGY TDRGWGNGCG

Muar (A)        LFGKGSIDTC AKLSCSSKAI GKIIQPENIK YEVGIFVHGT TTAENHGNYS
Muar (B)        LFGKGSIDTC AKFVCSHKAI GKIIQPENIK YEVGVFVHGT TTAENHGNYS

Muar (A)        AQVGASQAAK FTITPNAPSI TLKLGDYGEV TMDCEPRSGF NTEAFYVLTV
Muar (B)        AQIGASQAAK FTITPNAPSI TLKLGDYGEV TMDCEPRSGF NTEAFYVLTV

Muar (A)        GTKSFLVHRE WFNDLALPWL SPSSTNWRNR EILLEFEEAH ATKQSVVALG
Muar (B)        GTKSFLVHRE WFNDLALPWL SPSSTNWRNR EILLEFEEAH ATKQSVVALG

Muar (A)        SQEGALHQAL AGAIVVEYSS SVKLTSGHLK CRLKMDKLAL KGTTYGMCTE
Muar (B)        SQEGALHQAL AGAIVVEYSS SVKLTSGHLK CRLKMDKLAL KGTTYGMCTE

Muar (A)        KFSFSKNPAD TGHGTVVIEL QYTGTDGPCK IPISSVASLN DLTPVGRLVT
Muar (B)        KFSFSKNPAD TGHGTVVIEL QYTGTDGPCK IPISSVASLN DLTPVGRLVT
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Muar (A)          VNPFVATSTA NSKVLVELEP PFGDSFIVVG RGDKQINHHL HKVGSTLGKA
Muar (B)          VNPFVATSTA NSKVLVELEP PFGDSFIVVG RGDKQINHHW HKAGSSLGKA

Muar (A)          FSTTLKGAQR LAVLGDTDWD FGSTGGVFNS IGKAVHHVFG GTFRTLFGGM
Muar (B)          FTTTLKGAQR LAALGDTAWD FGSIGGVFNS IGKAVHQVFG GAFRTLFGGM

Muar (A)          SWITQGLMGA LLLWMGVKAR DGSIALAFLG TGGVLVFLAT NVHA
Muar (B)          SWITQGLMGA LLLWMGINAR DRSIALAFLA TGGVLLFLAT NVHA
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# SELECTED PRESENTATIONS AND PUBLICATIONS

# SELECTED PRESENTATIONS:

**27TH April 2010** **Mohammed, M. A.**, Siritorn Butrapet, Sareen E. Galbraith, Ichiro Kurane, Alan Radford & Tom Solomon..
***"Molecular Evolution of Japanese Encephalitis Virus"***,
University of Liverpool, School of Infection and Host Defence Away Day, Ness Botanic Gardens Neston, Wirral. (Poster)
This poster was ranked as the second best poster.

**31st March 2010** **Mohammed, M. A.**, Siritorn Butrapet, Sareen E. Galbraith, Ichiro Kurane, Alan Radford & Tom Solomon..
***"Molecular phylogenetic and evolutionary analyses of Muar strain of Japanese encephalitis virus: the missing 5th genotype"***.
Society of General Microbiology (SGM) Spring 2010 Meeting, Edinburgh International Conference Centre. (Talk)

**22nd January 2010** **Mohammed, M. A.**, Sareen E. Galbraith, Alan Radford & Tom Solomon
***"Origin and evolution of Japanese encephalitis virus".***
Workshop on Molecular evolution, Czech Republic, Europe. (Talk)

**1st April 2009** **Mohammed, M. A.**, Siritorn Butrapet, TomohikoTakasaki, Ichiro Kurane, Sareen E. Galbraith& Tom Solomon
***"Phylogenetic analysis of Muar strain of Japanese encephalitis virus reveals a possible 5th Genotype".***
Society of General Microbiology (SGM) Spring 2009 Meeting, Harrogate International Centre. (Talk)

I have also presented my PhD work at Liverpool Brain Infections Group's lab meetings and the departemental postgraduate seminars at University of Liverpool, United Kingdom.

# SELECTED PUBLICATIONS:

**Mohammed, M. A.**, S. E. Galbraith, Alan D. Radford, Winifred Dove, TomohikoTakasaki, Ichiro Kurane, Tom Solomon (2011). ***"Molecular phylogenetic and evolutionary analyses of Muar strain of Japanese encephalitis virus reveal it is the missing fifth genotype".*** Infection Genetics and Evolution 11 (5), 855-862.

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# Molecular phylogenetic and evolutionary analyses of Muar strain of Japanese encephalitis virus reveal it is the missing fifth genotype

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ABSTRACT

Japanese encephalitis virus (JEV) is the most important cause of epidemic encephalitis worldwide but its origin is unknown. Epidemics of encephalitis suggestive of Japanese encephalitis (JE) were described in Japan from the 1870s onwards. Four genotypes of JEV have been characterised and representatives of each genotype have been fully sequenced. Based on limited information, a single isolate from Malaysia is thought to represent a putative fifth genotype. We have determined the complete nucleotide and amino acid sequence of Muar strain and compared it with other fully sequenced JEV genomes. Muar was the least similar, with nucleotide divergence ranging from 20.2 to 21.2% and amino acid divergence ranging from 8.5 to 9.9%. Phylogenetic analysis of Muar strain revealed that it does represent a distinct fifth genotype of JEV. We elucidated Muar signature amino acids in the envelope (E) protein, including E327 Glu on the exposed lateral surface of the putative receptor binding domain which distinguishes Muar strain from the other four genotypes. Evolutionary analysis of full-length JEV genomes revealed that the mean evolutionary rate is $4.35 \times 10^{-4}$ ($3.4906 \times 10^{-4}$ to $5.303 \times 10^{-4}$) nucleotides substitutions per site per year and suggests JEV originated from its ancestral virus in the mid 1500s in the Indonesia-Malaysia region and evolved there into different genotypes, which then spread across Asia. No strong evidence for positive selection was found between JEV strains of the five genotypes and the E gene has generally been subjected to strong purifying selection.



## 1. Introduction

The genus *Flavivirus*, family *Flaviviridae* are important human pathogens that include a number of emerging and re-emerging pathogens such as Japanese encephalitis virus (JEV), West Nile virus (WNV), St. Louis encephalitis virus (SLEV), Kunjin virus (KUNV), Murray Valley encephalitis virus (MVEV), yellow fever virus (YFV) and dengue viruses (DENV). They cause a variety of diseases ranging from mild febrile illness to severe encephalitis and hemorrhagic fever (Mishra and Basu, 2008). The most important cause of epidemic encephalitis worldwide is JEV, with an estimated 35,000–50,000 cases and 10,000 deaths annually (Tsai, 2000).

JEV is a member of the JE serogroup of flaviviruses and is transmitted between vertebrate hosts by mosquitoes, principally by Culex species especially *Culex tritaeniorhynchus* (Yun et al., 2003). The virus is maintained mainly between vector mosquitoes and wading birds, with pigs also acting as amplifying hosts. Humans become infected during the bite of an infected mosquito.

JEV has a genome structure, which is similar to that of other flaviviruses. It is a small-enveloped virus with a single-stranded, positive-sense RNA genome of approximately 11 kb in length. The genome contains a single long open reading frame (ORF) flanked by 5′ and 3′ nontranslated regions (NTRs) that are important Cis-acting elements for viral replication. The RNA genome has a type I cap structure at its 5′ terminus but lacks a poly(A) tail at its 3′. The ORF is translated into a large polyprotein that is co- or post-translationally processed into three structural and seven non-structural proteins whose genes are arranged in the genome as follows: C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 (Chambers et al., 1990; Lindenbach and Rice, 2001; Venugopal and Gould, 1994).

Outbreaks of encephalitis suggestive of JE were recorded in Japan from the 1870s onwards and JEV was first isolated in Japan in

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1935 (Lewis et al., 1947). Subsequently, JEV has been found throughout most of Asia and the virus was most recently isolated in Papua New Guinea and the Torres Straits of Australia, indicating that this virus could become a worldwide public health threat (Hanna et al., 1996, 1999; Mackenzie et al., 1994). The geographical expansion of JEV's range appears to relate to irrigated rice fields and to a limited extent to pig farming. These environments that are suitable for infectious cycle of JEV exist widely in Asia (Oya and Kurane, 2007). The origins of the virus are uncertain, but phylogenetic comparisons with other flaviviruses suggest it evolved from an African ancestral virus, perhaps as recently as a few centuries ago (Gould, 2002). Two distinct epidemiological patterns of JEV transmission have been observed. In northern temperate regions of Asia JE occurs in summer epidemics, whereas in southern tropical areas the disease is endemic and occurs year-round (Vaughn and Hoke, 1992).

Based on genetic studies, JEV consists of four genotypes (I–IV) (Chen et al., 1990, 1992). Genotype I includes isolates from Northern Thailand, Cambodia, Korea, China, Japan, Vietnam, Taiwan, and Australia from 1967 to the present. Genotype II includes isolates from Southern Thailand, Malaysia, Indonesia, Papua New Guinea and Northern Australia from before 1951 to 1999. Genotype III includes isolates from mostly temperate regions of Asia, including Japan, China, Taiwan, the Philippines, and the Asian subcontinent from 1935 to the present, and genotype IV includes isolates from Indonesia between 1980 and 1981 only (Schuh et al., 2010). Representatives of each of these four genotypes have been fully sequenced.

The Muar strain of JEV was isolated in Singapore in 1952 from a brain of a patient who originated in Muar, Malaysia (Hale et al., 1952) and this strain may represent a fifth genotype as determined by monoclonal antibodies reactivities (Hasegawa et al., 1995; Kobayashi et al., 1984) and limited phylogenetic analysis based on E gene sequence (Hasegawa et al., 1994; Solomon et al., 2003; Uchil and Satchidanandam, 2001). Here we have determined the complete nucleotide and deduced amino acid sequence of the Muar isolate and its evolutionary history. We analyzed selection pressures for the complete E gene of representative strains of the five genotypes in order to identify the patterns of genetic change of JEV genotypes. We predicted the three-dimensional structure of envelope glycoprotein of Muar strain and observed the difference and critical changes between Muar and other JEV strains so this may help us to understand the variations in antigenic and neutralizing properties of different JEV strains.

## 2. Materials and methods

### 2.1. Culture and sequencing of Muar isolate

The Muar strain of JEV, isolated in Singapore in 1952 from the brain of a patient who originated in Muar, Malaysia, was inoculated onto confluent monolayers of kidney epithelial cells extracted from an African green monkey (Vero cells) in 5% fetal calf serum. Culture supernatant containing the virus was harvested at 5–6 days postinfection, when cytopathic effects were observed, and virus RNA was extracted by using the QIAamp Viral RNA Mini Kit (Qiagen). RNA was reverse transcribed and was PCR amplified by using Titan One Tube RT-PCR System (Roche). Primers were designed to amplify overlapping fragments of the genome (~1000 bp in length) on the basis of consensus alignment of complete JEV nucleotide sequences, and on partial sequences of Muar already derived by us and others (Hasegawa et al., 1994).

RT-PCR products were purified by using the QIAquick Gel Extraction Kit. Purified cDNA was directly sequenced in both directions by using the appropriate sense and antisense primers. Sequencing of the 5′ UTR was determined by using 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) and the 3′ UTR sequence was obtained by using *E. coli* poly(A) polymerase (BioLabs) to add a poly(A) tail to Muar mRNA. Subsequently, the poly(A) mRNA was reverse transcribed and was PCR amplified by HotStarTaq Master Mix Kit (Qiagen) using a gene-specific primer. If there were differences between sequence results, cloning of that region was performed using the TA Cloning® Kit (Invitrogen) and ten clones of plasmids containing inserts were sequenced in both directions by using Muar-specific forward and reverse primers. The complete genome of the Muar strain was compiled by alignment with other JEV complete genome strains (Table 1), using the Vector NTI suite software package (version 10; Informax Inc.). Percentage differences between the complete genomic sequence and the derived polyprotein of the Muar strain and representative strains of JEV genotypes I–IV were calculated by using P-distance in MEGA software (version 4.0).

### 2.2. Phylogenetic relationships of the Muar isolate with other JEV strains

ClustalX (1.81) was used for the alignment of Muar nucleotide and derived amino acid sequences with other JEV strains available at GenBank. Selection of the best-fit model for JEV genome nucleotide substitution was carried out by ModelTest 3.7 (version 0.1.1). Phylogenetic analysis was performed using maximum-parsimony and neighbour-joining methods on PAUP* (version 4.04b 10) and maximum-likelihood method on GARLI (version 0.96 Beta) using nucleotide and derived polyprotein sequences of C, PrM, E, NS3, NS5 and ORF as well as the nucleotide sequences of the 5′ UTR and 3′ UTR and the complete genome. The robustness of phylograms was evaluated by 1000 bootstrap replicates. All trees were visualised using FigTree (version 1.2.2).

### 2.3. Evolutionary history of Muar isolate

A Maximum Clade Credibility (MCC) tree based on 35 complete coding sequences of JEV was inferred using the Bayesian Markov Chain Monte Carlo (MCMC) method available in the BEAST package (version 1.5b2) (Drummond and Rambaut, 2007), thereby incorporating information on the isolates time of original sampling. This analysis utilized a relaxed molecular clock (uncorrelated lognormal) and a GTR + Gamma + Invariant model of nucleotide substitution for each codon position. All chains were run for a sufficient length to ensure convergence with 10% removed as burn-in. This analysis also allowed us to estimate coalescent (divergence) times for each node on the JEV phylogeny. The degree of uncertainty in each parameter estimate is provided by the 95% highest posterior density (HPD) values, while posterior probability values provide an assessment of the degree of support for each node on the tree.

The evolutionary rate of the complete genome between representative strains of GI and GIII, and for the envelope gene between the strains of GI, GII and GIII was estimated. Such analysis was not possible between the strains of GIV and GV as there is only one representative strain published on GenBank.

### 2.4. Selection pressures between genotypes

Overall and site specific selection pressures in the E genes of 66 JEV strains belonging to the five JEV genotypes were measured as the nonsynonymous (silent)/synonymous (amino acid-altering) rate ratio ($\omega = dN/dS$) using a maximum likelihood method implemented by Datamonkey (Pond and Frost, 2005). Tests for positive selection were performed using three methods – single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), and internal FEL (IFEL) (http://www.datamonkey.org).

**Table 1**
Details of isolates used for analysis in this study. All strains are JEV, except for MVE, SLE and WN.

| Strain | Year | Location | Source | GenBank accession no. |
|---|---|---|---|---|
| 014178 | 2001 | India | Human | EF623987 |
| 04940-4 | 2002 | India | Mosquito | EF623989 |
| 057434 | 2005 | India | Human | EF623988 |
| 2372 | 1979 | Thailand | Human | U70401 (E) |
| 691004 | 1969 | Sri Lanka | Human | Z34097 (E) |
| B58 | 1986 | China | Bat | FJ185036 |
| Beijing-1 | 1949 | China | Human | L48961 |
| Bennett | Before 1951 | Korea | Human | FJ872376 (E) |
| CH1392 | 1990 | Taiwan | Mosquito | AF254452 |
| CH2195LA | 1994 | Taiwan | Mosquito | AF221499 |
| CNS138-11 | 1999 | Malaysia | Human | AY184213 (E) |
| FU | 1995 | Australia | Human | AF217620 |
| GB30 | 1997 | China | Bat | FJ185037 |
| GP05 | 2005 | India | Human | FJ979830 (E) |
| GP78 | 1978 | India | Human | AF075723 |
| HEN0701 | 2007 | China | Swine | FJ495189 |
| HVI | 1958 | Taiwan | Human | AF098735 |
| Ishikawa | 1998 | Japan | Mosquito | AB051292 |
| JaGAr01 | 1959 | Japan | Mosquito | AF069076 |
| JaNAr0102 | 2002 | Japan | Mosquito | AY377577 (E) |
| JaOArS982 | 1982 | Japan | Mosquito | NC_001437 |
| JaOH0566 | 1966 | Japan | Human | AY508813 |
| JEV/sw/Mie/40/2004 | 2004 | Japan | Swine | AB241118 |
| JEV/sw/Mie/41/2002 | 2002 | Japan | Swine | AB241119 |
| JKT1724 | 1979 | Indonesia | Mosquito | U70404 (E) |
| JKT5441 | 1981 | Indonesia | Mosquito | U70406 (E) |
| JKT6468 | 1981 | Indonesia | Mosquito | AY184212 |
| JX61 | 2008 | China | Pig | GU556217 |
| K87P39 | 1987 | Korea | Mosquito | AY585242 |
| K94P05 | 1994 | Korea | Mosquito | AF045551 |
| KPP034-35CT | 1982 | Thailand | Mosquito | U03693 (E) |
| Ling | 1965 | Taiwan | Human | L78128 |
| M15 | 1995 | Australia | Mosquito | L47349 (E) |
| M40 | 1995 | Australia | Mosquito | L47350 (E) |
| M859 | 1967 | Cambodia | Mosquito | U70410 (E) |
| Muar | 1952 | Malaysia | Human | This paper, accession no. HM596272 |
| MVE 1-51 | 1951 | Australia | Human | AF161266 |
| Nakayama | 1935 | Japan | Human | EF571853 |
| Nakayama- RFVL | IU | Japan | IU | S75726 (E) |
| NJ 2008 | 2008 | IU | IU | GQ918133 |
| NO | 1995 | Australia | Human | L43566 (E) |
| P20778 | 1958 | India | Human | AF080251 |
| P3 | 1950 | China | Mosquito | U47032 |
| PhAn1242 | 1984 | Philippines | Pig | U70417 (E) |
| RP-9 | 1985 | Taiwan | Mosquito | AF014161 |
| SA14 | 1954 | China | Mosquito | U14163 |
| SC04-17 | 2009 | China | Mosquito | GU187972 |
| SH17M07 | 2007 | China | IU | EU429297 |
| SLE MSI.7 | 1975 | United States | Bird | NC_007580 |
| T1P1 | 1997 | Taiwan | Mosquito | AF254453 |
| TC | IU | Taiwan | IU | AF098736 |
| TS00 | 2000 | Australia | Pig | EF434785 (E) |
| VN118 | 1979 | Vietnam | Mosquito | U70420 (E) |
| WN Eg101 | 1950 | Egypt | Human | AF260968 |
| WTP-70-22 | 1970 | Malaysia | Mosquito | U70421 (E) |
| XJ69 | 2007 | China | Mosquito | EU880214 |
| XJP613 | 2007 | China | Mosquito | EU693899 |

GenBank accession numbers refer to the whole genome or the genes indicated in parentheses. E, envelope gene. IU, information unavailable.

### 2.5. Prediction of three-dimensional structure of envelope glycoprotein of Muar strain

Muar-specific amino acids were identified by comparing the E glycoprotein sequence to 292 other JEV strain sequences. These were subsequently mapped onto the predicted 3D structure of the Muar envelope protein E, as modelled based on predicted similarities to the homologous (77% identity) glycoprotein gene in WNV (PDB entry 2i69) using SWISS-MODEL via the ExPASy web server (Schwede et al., 2003). The predicted Muar E structure was viewed and manipulated using PyMol (http://pymol.sourceforge.net/). Critical changes in the E glycoprotein which are thought to be involved in receptor binding as described by Ni and Barrett (1998) and Solomon et al. (2003) were identified.

### 2.6. Nucleotide sequence accession number

The JEV isolate Muar was assigned GenBank database accession no. HM596272.

## 3. Results

### 3.1. Nucleotide and amino acid sequence

The Muar virus genome was 10,988 nucleotides long. Comparison with other fully sequenced JEV genomes showed Muar to have the least similarity, with nucleotide divergence ranging from 20.2 to 21.2%, and protein divergence from 8.5 to 9.9% (Table 2).

**Table 2**
Sequence divergence among representatives of the five genotypes of JEV complete genomes.

| Strain | Genotype | Divergence (%) with | | | | |
|---|---|---|---|---|---|---|
| | | K94P05 | FU | Nakayama | JKT6468 | Muar |
| K9P05 | I | | 10.3 | 10.8 | 16.8 | 21.2 |
| FU | II | **2.1** | | 10.5 | 16.1 | 21.1 |
| Nakayama | III | **3.1** | **2.2** | | 15.1 | 20.2 |
| JKT6468 | IV | **6.2** | **5.3** | **4.8** | | 21.0 |
| Muar | V | **9.9** | **8.6** | **8.5** | **9.2** | |

The nucleotide divergence is shown in lightface type and the amino acid divergence is shown in bold face type.

Although the sequence of the structural genes of the Muar strain was previously published by Hasegawa et al. (1994) but we identified a number of nucleotide differences between their sequence and our sequence (genetic distance 6.7%).

### 3.2. Phylogenetic analyses and evolutionary history of Muar

Maximum likelihood phylogenetic analysis of 41 E genes (1500 bp) and 34 NS5/3′ UTR (3380 bp) sequences for JEV and representative strains of other viruses in the JE serogroup shows that the Muar isolate is a fifth genotype of JEV (Fig. 1A and B). Similar trees, with 100% bootstrap support, were produced by the maximum-parsimony, neighbour-joining and maximum likelihood methods.

Molecular clock analysis of 35 complete coding sequences of JEV, supported by high posterior probability values, confirmed that Muar belongs to a fifth JEV genotype and represents the oldest linage of JEV, which last shared a common ancestor from 449.6 years ago (95% HPD = 373.9–499.9) (Fig. 2).

Evolutionary analysis of 35 full-length JEV genomes revealed that the mean evolutionary rate of JEV isolates is $4.35 \times 10^{-4}$ (95% HPD = $3.4906 \times 10^{-4}$ to $5.303 \times 10^{-4}$) nucleotides substitutions per site per year. While the mean evolutionary rate between 10 strains of GI, and 22 strains of GIII complete genomes are $3.029 \times 10^{-4}$ (95% HPD = $2.0884 \times 10^{-8}$ to $8.6999 \times 10^{-4}$) and $6.029 \times 10^{-4}$ (95% HPD = $7.0492 \times 10^{-8}$ to $1.4093 \times 10^{-4}$) nucleotides substitutions per site per year, respectively. Comparing the mean evolutionary rate of each genotype separately using envelope genes showed that the mean evolutionary rate between 12 strains of GI, 7 strains of GII and 39 strains of GIII are $1.14 \times 10^{-3}$ (95% HPD = $2.3 \times 10^{-4}$ to $2.2 \times 10^{-3}$), $1.97 \times 10^{-3}$ (95% HPD = $2.8 \times 10^{-4}$ to $3.95 \times 10^{-3}$) and $2.69 \times 10^{-4}$ (95% HPD = $1.09 \times 10^{-4}$ to $4.20 \times 10^{-4}$) nucleotides substitutions per site per year, respectively (Table 3).

### 3.3. Selection pressures between the five JEV genotypes

There was no evidence for positive selection between the JEV five genotypes strains and different selection pressures models did not have any category with $\omega > 1$. The average $\omega$ ratio ($dN/dS$) over the entire E gene sites was estimated to be ~0.05, suggestive of predominantly purifying selection.

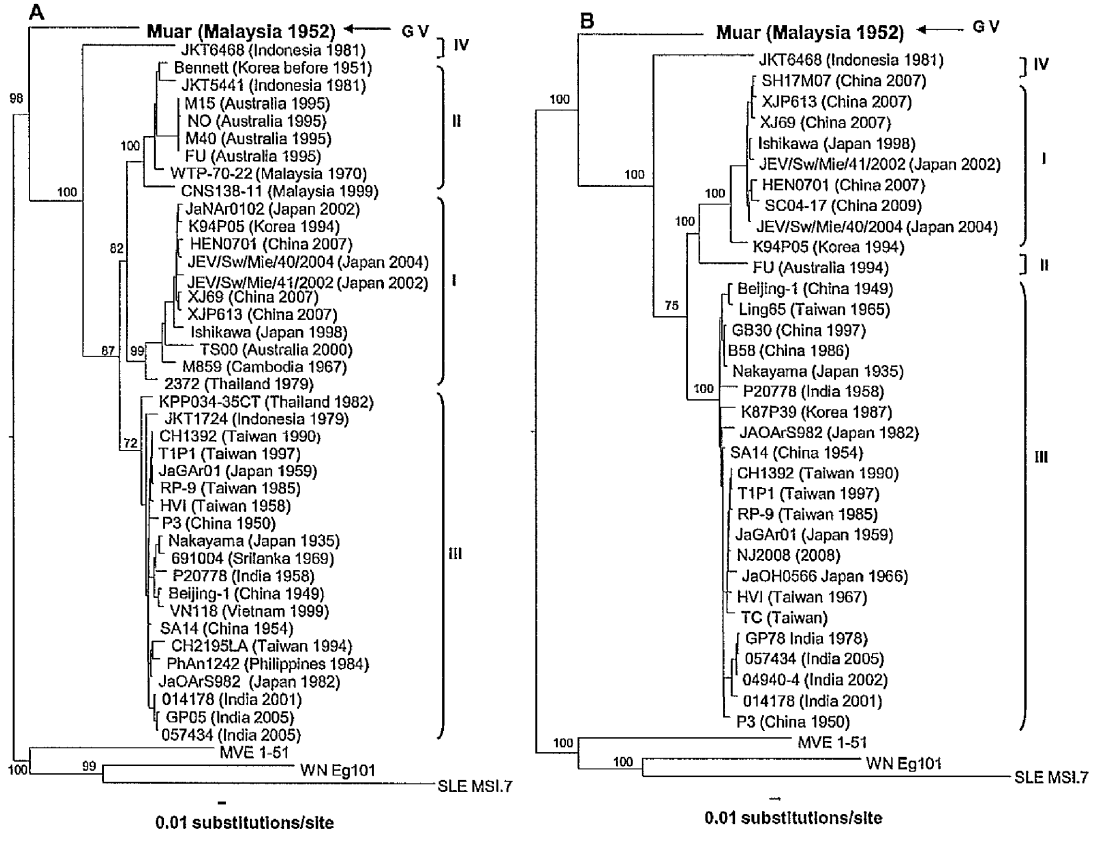


**Fig. 1.** Maximum likelihood phylogenetic trees of the (A) envelope genes and (B) NS5/3′ UTR genes, outgrouped by using a representative strain from other viruses in the JEV serogroup (MVE, Murray Valley encephalitis; SLE; St. Louis encephalitis; WN, West Nile). The Muar isolate is shown with an arrow as a genotype V. Genotypes are given on the right of each tree. Bootstrap support values, given as a percentage of 1000 replicates, are shown.

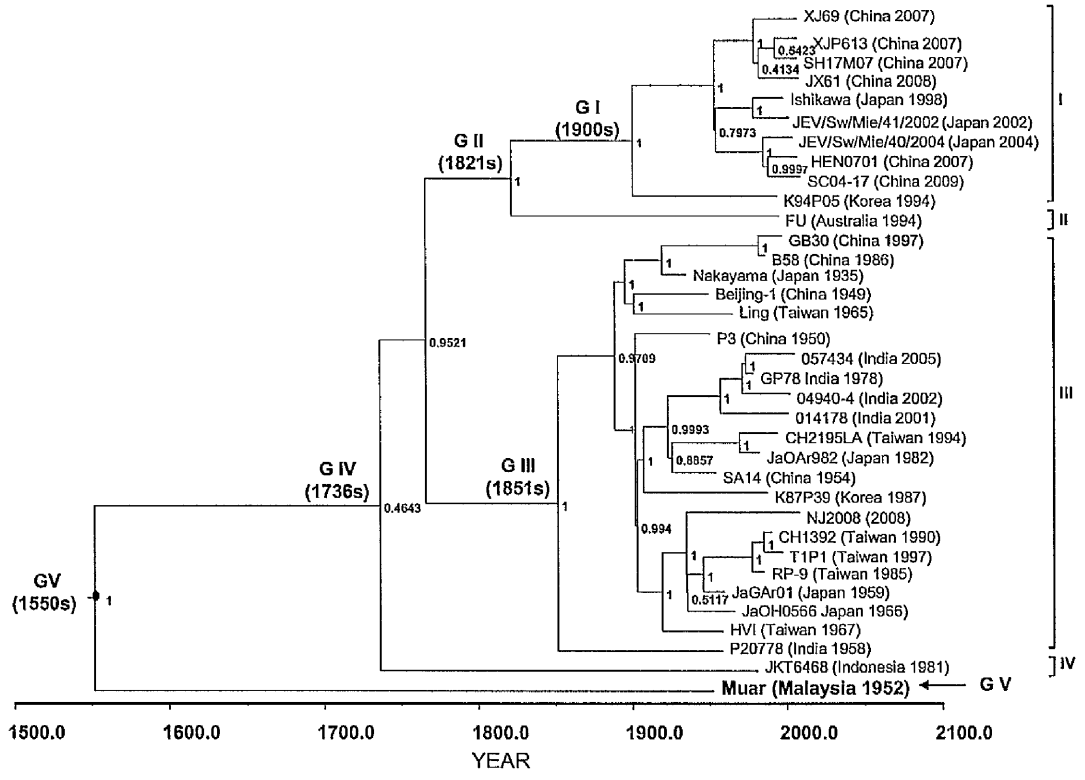


Fig. 2. Maximum clade credibility (MCC) phylogeny for 35 complete coding sequences of JEV genomes. Horizontal branches are drawn to a scale of estimated year of divergence with tip times reflecting sampling date (year). The tree is automatically rooted due to the assumption of a molecular clock. The divergence times of genotype nodes are shown. High posterior probability values are shown beside the nodes. A filled black circle identifies the most recent common ancestor of JEV. Muar isolate, the oldest linage, is shown with an arrow as a genotype V.

### 3.4. Structural differences

Examination of the E protein of Muar showed it was similar to other flaviviruses, with 12 cysteine residues that form six disulfide bridges. Alignment of the Muar E protein amino acid sequence with representative strains from each genotype, GI (K94P05 and XJP613), GII (Bennett, CNS138-11 and FU), GIII (691004, Nakayama-RFVL and P3), GIV (JKT6468) and GV (Muar), is shown in Fig. 3. The alignment of E genes from 292 strains identified 22 signature amino acid residues that distinguished genotype V from other genotypes. The range of intergenotypic amino acid sequence divergence of E protein between the genotype V strain, Muar, and the other four JEV genotypes is as follows: Muar versus genotype I (8.9–9.1%), genotype II (8.5–9.1%), genotype III (8.7–9.7%) and genotype IV (9.5%).

**Table 3**
Rates of nucleotide substitutions per site for JEV complete genomes and envelope genes.

| JEV genotype | Mean no. of nucleotide substitutions/site/year | 95% HPD | |
|---|---|---|---|
| | | Lower | Upper |
| GI-V complete genomes | $4.35 \times 10^{-4}$ | $3.4906 \times 10^{-4}$ | $5.303 \times 10^{-4}$ |
| GI complete genomes | $3.029 \times 10^{-4}$ | $2.0884 \times 10^{-8}$ | $8.6999 \times 10^{-4}$ |
| GIII complete genomes | $6.029 \times 10^{-4}$ | $7.0492 \times 10^{-8}$ | $1.4093 \times 10^{-4}$ |
| GI envelope genes | $1.14 \times 10^{-3}$ | $2.3 \times 10^{-4}$ | $2.2 \times 10^{-3}$ |
| GII envelope genes | $1.97 \times 10^{-3}$ | $2.8 \times 10^{-4}$ | $3.95 \times 10^{-3}$ |
| GIII envelope genes | $2.69 \times 10^{-4}$ | $1.09 \times 10^{-4}$ | $4.20 \times 10^{-4}$ |

The predicted three-dimensional structure of the E protein of Muar strain was modelled onto the crystal structure of WNV (Nybakken et al., 2006) and showed two signature amino acids for Muar in domain I, twelve in domain II and eight in domain III. This included a critical change identified in the E protein at position 327 at the exposed lateral surface of domain III in a region thought to be involved in receptor binding as described previously (Ni and Barrett, 1998; Solomon et al., 2003) (Fig. 4). This critical change is from glutamine in genotype V strain to leucine in genotype IV strains then to serine and threonine amino acids found in newer genotypes – I, II and III.

## 4. Discussion

Arthropod-borne encephalitis viruses represent a significant public health problem throughout most of the world but their origins and evolution remain unclear (Solomon and Cardosa, 2000). JEV was first described in Japan in 1870 and subsequently appeared to spread across Asia to affect most of China and the Asian subcontinent, all of Southeast Asia, and the Pacific Rim, reaching northern Australia in 1998. JEV has become the most important cause of epidemic encephalitis worldwide and there is a clear need for a better understanding of the origins and spread of the virus. Epidemiological studies have demonstrated four genotypes of JEV (I–IV) and at least five antigenic subgroups of JEV have circulated in Asia since isolation of the original Nakayama virus in 1935 (Hasegawa et al., 1994; Kedarnath et al., 1986; Kobayashi et al., 1984, 1985). One of these antigenic subgroups includes only the Nakayama-derived strains, whereas the majority of the more recent isolates in genotype III are within a subgroup

Dots indicate consensus. Underlined amino acids represent Muar signature amino acids.

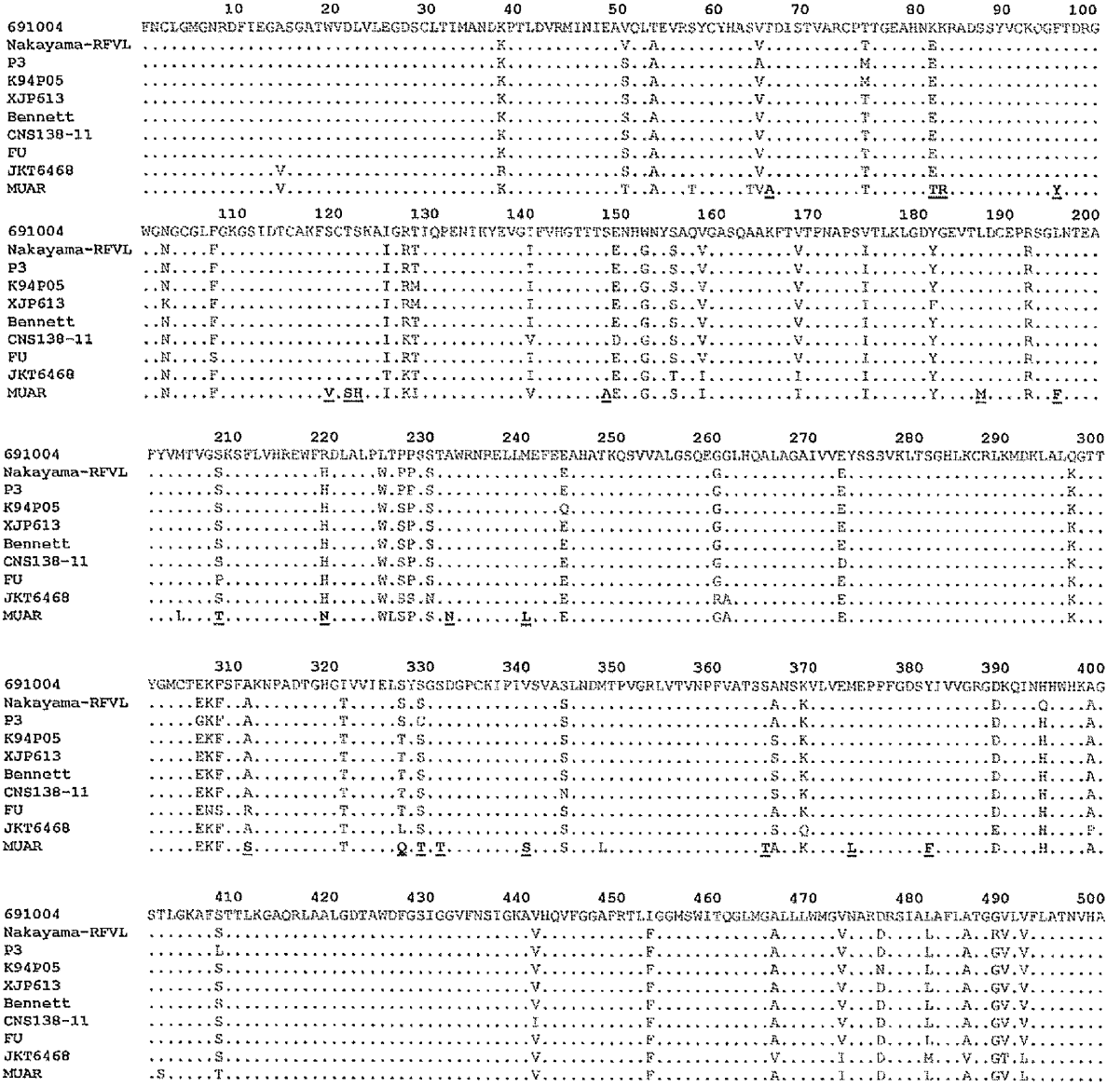


Fig. 3. Alignment of the E protein amino acid sequences of JEV G I–V. Dots indicate consensus. Underlined amino acids represent Muar signature amino acids.

containing the Kamiyama strain isolated in 1966 (Kobayashi et al., 1985). In addition, two antigenic subgroups are quite distinct, the 691004 (isolated in Sri Lanka in 1969) and Muar (isolated in Singapore in 1952) subgroups, each containing only the prototype virus for the respective subgroup (Kobayashi et al., 1985).

Sequencing of the Muar strain complete genome showed it was the most genetically different from other JEV strains, in agreement with serological results using anti-Muar monoclonal antibodies (Hasegawa, 1982; Hasegawa et al., 1995), and confirming that Muar is unique both genetically and serologically. Using the classification of Chen et al. (1992), Ali and Igarashi (1997), and Uchil and Satchidanandam (2001), our phylogenetic analyses clearly support the evidence that Muar represents a fifth genotype of JEV.

Hasegawa et al. (1994) previously published a sequence for the structural genes of the Muar isolate, and a number of nucleotide differences between their sequence and our sequence were identified (genetic distance 6.7%). The reasons for these differences are difficult to explain. Some differences are to be expected due to variability arising during growth of the RNA viruses in culture and PCR steps. Some differences may also be due to the differences in sequencing strategies; Hasegawa et al. (1994) used manual sequencing, whereas we used modern sequencing protocols. Alternatively, it is possible that the older sequencing methods produced results that were less reliable, or differences in the passage history since original isolation of the virus explain the sequence differences.

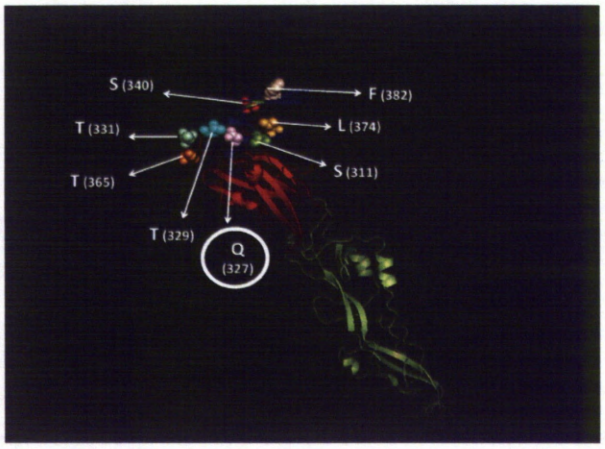


**Fig. 4.** JEV envelope protein. Predicted three-dimensional model of the E protein of JEV genotype V strain Muar, derived from the crystal structure of West Nile virus, showing 8 Muar signature amino acids in domain III with the critical change at residue 327; circled (in the putative receptor binding region of domain III).

Our analyses suggest that the Muar strain represents the oldest JEV lineage when compared with other genomic sequences. Because of this we examined the geographical distribution of all known isolates. Evolutionary analysis suggests JEV originated from its ancestral virus in the mid 1500s in the Indonesia-Malaysia region and evolved there into different genotypes, which then spread across Asia. Estimates of divergence times of JEV genotypes suggested that the deepest divergence of known JEV strains was at 1550s. Although our results agree with the previous studies which suggest that JEV arose from its ancestral virus in the Indonesia-Malaysia region and evolved into five genotypes; genotypes IV and V, the most divergent genotypes, remain confined to the Indonesia-Malaysia region, whereas genotypes I, II and III, the most recent evolved genotypes appear to have spread across Asia (Solomon et al., 2003), one cannot be absolutely certain that this is the case.

Evolutionary analysis of full-length JEV genomes showed high mean evolutionary rate ($4.35 \times 10^{-4}$) which is expected for JEV as an RNA virus which lacks a proof reading mechanism during replication. Evolutionary rate of envelope genes for each genotype separately showed a higher evolutionary rate for genotypes I and II than genotype III.

Understanding these genetic changes in the E gene is an important step to study the evolution of JEV. The extensive surveillance helps us understand geographic movement and genotype shift of the virus. We determined the selection pressures on the E gene sequences of the five genotypes. Many sites seemed to be under negative selection pressure, and no evidence for positive selection was found. The intensity of selection is reflected in the magnitude of $dN/dS$ ratio. Indeed, the overall picture obtained was that of strong purifying selection, with mean $dN/dS$ value ~0.05 for all JEV genotypes, and similar to the previous studies by Tang et al. (2010). This suggests that the antigenic selection might not be the reason for genotype shift of JEV.

The observed difference in the predicted three dimensional structure of E glycoprotein of JEV strains, particularly in domain III, could help in understanding the antigenic and neutralizing properties of JEV strains (Cecilia and Gould, 1991; Gritsun et al., 1995). Hence, Domain III of the E protein of JEV has been shown to contain important sites for antibody-mediated virus neutralization and motifs associated with virulence (Rey et al., 1995; Wu et al., 2003); the eight signature amino acids differences at domain III with a critical change at residue 327 (in the putative receptor binding region of domain III), may explain some of the serological differences with Muar strain.

In summary, our complete genome sequence analysis confirms that the Muar strain represents a fifth genotype of JEV with approximately 20% divergence from other JEV strains. Evolutionary analysis suggested Muar represents the oldest JEV lineage and the four genotypes diverged from genotype V approximately 450 years ago. Whether there are other strains from genotype V, and what happened to them remains unknown.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.01.020.

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